MECHANISMS OF POTASSIUM PERMEATION IN THE *IN VITRO* PERFUSED CORTICAL COLLECTING DUCT OF THE RABBIT

By

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A DISSERTATION PRESENTED TO THE GRADUATE SCHOOL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1992

To Xiaofang, Martin, my parents, and my brother.

TABLE OF CONTENTS

ACKNOWLEGEMENTS v

LIST OF TABLES viii

LIST OF FIGURES x

KEY TO ABBREVIATIONS xiii

ABSTRACT xv

CHAPTER 1 INTRODUCTION 1

1.1 Background 1
1.2 Potassium Secretion by the Cortical Collecting Duct 4
1.3 Potassium Absorption by the Cortical Collecting Duct . . . 10
1.4 The Aims and Objectives of the Present Studies 13

CHAPTER 2 GENERAL METHODOLOGY 23

2.1 *In Vitro* Microperfusion 23
2.2 Flameless Atomic Absorption Spectrophotometry 25
2.3 Statistical Analyses 26

CHAPTER 3 EFFECT OF 10% CO_2 ON Rb EFFLUX 28

3.1 Introduction 28
3.2 Methods and Materials 30
3.3 Results 31
3.4 Discussion 38
3.5 Summary 48

CHAPTER 4 EFFECT OF BARIUM, AMILORIDE AND OUABAIN ON Rb EFFLUX 70

4.1 Introduction 70
4.2 Methods and Material 72
4.3 Results 72
4.4 Discussion 76
4.5 Summary 79

CHAPTER 5 EFFECTS OF LUMINAL SODIUM ON Rb EFFLUX . 88

5.1 Introduction 88
5.2 Methods and Material 88
5.3 Results 89
5.4. Discussion 94
5.5 Summary 97

CHAPTER 6 EFFECT OF ANGIOTENSIN II, HISTAMINE, AND CARBACHOL 111

6.1 Introduction 111
6.2 Methods and Material 111
6.3 Results 112
6.4 Discussion 114
6.5 Summary 115

CHAPTER 7 EFFECT OF Na AND K INTAKE ON SERUM AND URINE Na AND K LEVELS AND URINE OUTPUT 122

7.1 Introduction 122
7.2 Methods and Material 122
7.3 Results 122
7.3 Discussion 123

CHAPTER 8 SUMMARY AND CONCLUSIONS 127

REFERENCES 137

BIOGRAPHICAL SKETCH 150

ACKNOWLEDGEMENTS

I wish to express my gratitude and appreciation to Dr. Charles S. Wingo, the chairman of my supervisory committee, for his instrumental guidance, productive encouragement, invaluable support and kind care throughout the fulfillment of this work. His tremendous contribution to my scientific knowledge, professional career, my personality and personal life are beyond my ability to acknowledge. I will benefit from his tradition of dedication, criticism, responsibility, preciseness and carefulness in both of my academic activities and private life for a very long time to come. I am indebted to Dr. George A. Gerencser, the co-chair of my committee for his vital role in my doctoral education. His thoughtful instruction was invaluable during the course of my studies. His understanding and friendship are greatly appreciated.

I extend my sincere thanks to the members of my supervisory committee, Drs. Lal C. Garg, Bruce R. Stevens and Brian D. Cain for their very helpful suggestions and criticism. They have provided me with alternative interpretations and additional insight that have required additional studies. I am grateful to Scott Straub and Amy Wall for their expert technical help when I was learning *in vitro* microperfusion. The personal friendship with Scott Straub and Harold Snellen established during past four years is warmly appreciated. I sincerely appreciate Dr. Frances Armitage and Wei Ueberschaer for their important role

in this research and for their help with my English. The discussions of my research results with them and with Drs. Kevin Curran and Mitchell Hebert have been productive and enjoyable. I am also grateful to the faculty members in the Department of Physiology, especially Drs. M. Ian Phillips, Colin Sumners, Sidney Cassin, Melvin Fregly and Charlie Wood for their advice and encouragement. In addition, I wish to thank Drs. C. Craig Tisher, Christopher Wilcox, Kirsten Madson, William Welch, and I David Weiner in the Division of Nephrology, Hypertension and Transplatation for their concern and advice. I wish to thank Ann Crawford in depth for her excellent and courteous help in preparation of this thesis as well as other manuscripts and abstracts. I am always grateful to have the manuscripts back from Ann before Friday so that I have something to work with during the weekend. My sincere thanks should be presented to Robert Fleming for his constant concern and support. My special thanks should be offered to Janice Dolson not only for her expert help with my learning some special functions of Wordperfect, but also for her friendly understanding and support during the past three years. I also appreciate Ms. Ginny Young for her secretarial assistance to Dr. Wingo regarding my education. My deep thanks are extended to Miss. B. J. Streetman, Victoria La Placa, Pia Jacobs and Gayle Butters and Mr. Kevin Fortin for their necessary role in my doctoral education. The constant concern and support from my fellow students in the Department of Physiology, especially Hong-gen Chen and Jian Kane, and Jenny Zhang in the Department of Pharmacology and Therapeutics are warmly appreciated.

I am greatly indebted to my wife Xiaofang, my father Quibao Zhou, my

mother Suping Bu and my brother Xiaolin for their love, consideration, backing and understanding. Xiaofang has unselfishly devoted her life to the family. This dissertation is especially dedicated to Xiaofang and Martin at this special time, although it is impossible to express my gratitude to them adequately in words. The birth of Martin in the final stage of my doctoral education not only has increased my excitement for the present studies, but also has inspired me to aim at higher quality of professional and private life in order for me to carry out the honor, duty, desire, and role model as a father. Without the fundamental and instrumental education of my childhood by my parents, it would be unimaginable for me to be able to choose the present way of pursuing my life.

LIST OF TABLES

Table 3-1. Composition of solution 50

Table 3-2. Effect of luminal 0.1% DMSO on K_{Rb} and V_T 51

Table 3-3. Time control for K_{Rb} and V_T 52

Table 3-4. Effect of 10% CO_2 on V_T in the presence colchicine . 53

Table 3-5. Effect of 10% CO_2 on V_T in the tubules pretreated with 0.5μM MAPTAM and W-7 54

Table 3-6. Effect of 10% CO_2 on K_{Rb} and V_T by the normal CCD in the presence and absence of luminal Ba 55

Table 4-1. Composition of solutions 80

Table 4-2. Effect of DMSO in the absence of luminal Na and luminal 1.5 mM Na 81

Table 4-3. Effect of luminal 10μM SCH28080 or 0.1 mM ouabain on V_T in the absence of luminal Na 82

Table 5-1. Composition of solution 99

Table 5-2. Effect of removal of luminal Na in the absence of luminal Ba, and of luminal Ba addition in the absence of luminal Na 101

Table 5-3. Effect of luminal Ba addition in the presence of luminal Na, and of luminal Na removal in the presence of luminal Ba 102

Table 5-4. Effect of removal of luminal Na in the presence of 4 mM Ba 103

Table 5-5. Effect of luminal Na removal of in the presence of both 2mM Ba and 10μM SCH28080 104

Table 5-6. Effect of luminal SCH28080 on V_T in the absence and

presence of luminal Na 105

Table 5-7. Effect of SCH28080 in the presence of 20 mM K . . . 106

Table 6-1. Composition of solution 116

Table 6-2. Effect of histamine on V_T in the presence of luminal 3 mM Ba 117

Table 6-3. Effect of 0.1 mM carbachol 118

Table 7-1. Effect of low Na and low K on excretion of urine, Na and K 125

Table 7-2. Effect of low Na and low K diet, and low K diet on serum Na and K levels 126

LIST OF FIGURES

Figure 1-1. Distribution of potassium in the body 16

Figure 1-2. Segmental analysis of tubule potassium transport 17

Figure 1-3. Urinary potassium excretion as a function of plasma potassium concentration in control animals and in alkalotic and acidotic conditions 18

Figure 1-4. Effect of different types of acid-base disturbances on potassium secretion by distal tubule 19

Figure 1-5. The cellular models of the principal cell and the intercalated cell 20

Figure 1-6. Cell model of epithelial sodium and potassium transport 21

Figure 1-7. The chemical structure of SCH28080 22

Figure 2-1. The cortical collecting duct perfused *in vitro* 27

Figure 3-1. Effect of 10% CO_2 on K_{Rb} and V_T in the absence of SCH28080 56

Figure 3-2. The predicted voltage-mediated increase in K_{Rb} and observed increase in K_{Rb} following exposure to 10% CO_2 57

Figure 3-3. Effect of 10% CO_2 on K_{Rb} and V_T in the presence of SCH28080 58

Figure 3-4. Effect of 10% CO_2 and 0.1 mM methazolamide on K_{Rb} and V_T in the absence of SCH28080 59

Figure 3-5. Effect of 10% CO_2 and 0.1 mM methazolamide on K_{Rb} and V_T in the presence of SCH28080 60

Figure 3-6. Effect of simultaneous exposure to 10% CO_2 and 0.1 mM methazolamide on K_{Rb} and V_T 61

Figure 3-7. Effect of 0.1 mM methazolamide on K_{Rb} and V_T in the presence of 5% CO_2 62

Figure 3-8. Effect of simultaneous exposure to 10% CO_2 and 0.5 mM colchicine and exposure to colchicine after 10% CO_2 on K_{Rb} 63

Figure 3-9. Effect of 10% CO_2 on K_{Rb} by the tubules pretreated with 0.5 μM MAPTAM and W-7 64

Figure 3-10. Effect of 10% CO_2 on K_{Rb} by the tubules pretreated with peritubular 3 mM Ba 65

Figure 3-11. The dose-response curve of Ba on K_{Rb} 66

Figure 3-12. The CCD was swollen following exposure to 10% CO_2 67

Figure 3-13. The simultaneous presence of 10% CO_2 and colchicine prevented the cell swelling 68

Figure 3-14. Effect of 10% CO_2 on cell swelling in the presence of MAPTAM 69

Figure 4-1. Effect of luminal 1 mM amiloride on K_{Rb} in the absence of luminal Ba and in the presence of luminal Ba, and effect of SCH28080 on K_{Rb} in the presence of luminal 1 mM amiloride and 2 mM Ba 83

Figure 4-2. Effect of luminal 1 mM amiloride on V_T in the absence of luminal Ba and in the presence of luminal Ba, and effect of SCH28080 on V_T in the presence of luminal 1 mM amiloride and 2 mM Ba 84

Figure 4-3. Effect of peritubular 0.1 mM ouabain on K_{Rb} in the absence of luminal SCH28080 and in the presence of luminal SCH28080 85

Figure 4-4. Effect of peritubular 0.1 mM ouabain on K_{Rb} in the absence of luminal SCH28080 and in the presence of luminal SCH28080 86

Figure 4-5. Effect of luminal SCH28080 or ouabain on K_{Rb} 87

Figure 5-1. Effect of lumen Na removal on K_{Rb} and V_T in the presence of luminal 10 μM SCH28080 107

Figure 5-2. Inhibition of K_{Rb} by luminal 10 μM SCH28080 in the presence of luminal Na and in the absence of luminal Na 108

Figure 5-3. Effect of SCH28080 on K_{Rb} in the presence of ambient 0.5 mM K or 20 mM K 109

Figure 5-4. Effect of SCH28080 on V_T in the presence of ambient 0.5 mM K or 20 mM K 110

Figure 6-1. Effect of angiotensin II on K_{Rb} and V_T in the presence of luminal 3 mM Ba and absence of luminal SCH28080 119

Figure 6-2. Effect of angiotensin II on K_{Rb} and V_T in the presence of luminal 10 μM SCH28080 and absence of luminal Ba 120

Figure 6-3. Effect of histamine on K_{Rb} in the presence of luminal 3 mM Ba 121

KEY TO ABBREVIATIONS

Ang II	angiotensin II
ANOVA	analysis of variance
ATP	adenosine triphosphate
Ba	barium
Ca	calcium
CCD	the cortical collecting duct
CD	the collecting duct
CO_2	carbon dioxide
H	proton
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
K	potassium
K_{Rb}	^{86}Rb lumen-to-bath efflux coefficient
K_{Na}	^{22}Na lumen-to-bath efflux coefficient
MAPTAM	Bis-(2-amino-5-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acidtetraacetoxymethyl ester N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide
Na	sodium
Rb	rubidium
SCH28080	(3-cyanomethyl-2-8-phenylmethoxy)imidazol[1,2-a]pyridine

TMA	tetramethylammonium
V_T	transepithelial voltage
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide

Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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May 1992

Chairman: Charles S. Wingo, M.D.
Major Department: Physiology

These studies were designed to examine the mechanisms of potassium (K) permeation across rabbit cortical collecting duct (CCD) assessed as ^{86}Rb lumen-to-bath efflux coefficient (K_{Rb}, or Rb efflux). This segment is primarily responsible for potassium secretion, but recent studies have demonstrated potassium absorption by the CCD.

The first set of studies was designed to examine whether acute peritubular acidosis (10% CO_2) stimulates rubidium (Rb) efflux, and whether this stimulation is dependent on the presence of functional H-K-ATPase, carbonic anhydrase, microtubules, the increase of intracellular calcium activity, calmodulin, and basolateral potassium conductance in the cortical collecting duct from K-restricted rabbits. Exposure to 10% CO_2 substantially stimulated Rb efflux, and this stimulation was totally abolished by 10μM SCH28080, a specific inhibitor of H-K-ATPase. After stimulation of Rb efflux by 10% CO_2,

subsequent addition of methazolamide or colchicine failed to affect Rb efflux. However, simultaneous exposure to 10% and methazolamide or colchicine prevented the stimulation of Rb efflux. Treatment with MAPTAM, W-7, and peritubular barium blocked the stimulatory effect of 10% CO_2. The data are consistent with the following conclusions, at least during K restriction: 1) an existing H-K-ATPase mediates the stimulatory effect of 10% CO_2 on Rb efflux; 2) carbonic anhydrase and microtubules are not necessary for maintaining H-K-ATPase activity, but is required for activation of H-K-ATPase by 10% CO_2; 3) the effect 10% CO_2 is dependent on the increase in intracellular calcium activity and presence of basolateral barium-sensitive exit mechanism.

The second set of the studies was mainly to examine the effect of luminal amiloride on Rb efflux. Amiloride significantly increased Rb efflux, and this effect was fully blocked by luminal barium, suggesting that a Ba-sensitive K conductance mediates the effect of amiloride.

The third set of the studies was to examine whether removal of luminal sodium had similar effect as amiloride. In contrast to the effect of amiloride, the stimulation of Rb efflux by sodium removal was only inhibited by simultaneous presence of barium and SCH28080, indicating that the effect of Na removal is in part via the H-K-ATPase. To test whether Na acts as a partial agonist of the H-K-ATPase we examined the effect of SCH28080 on Na efflux. In the presence of luminal amiloride and an ambient K concentration of 0.5 mM, SCH28080 significantly inhibited Na efflux, whereas this effect was abolished when similar experiments were performed in the presence of 20 mM ambient K. In conclusion: 1) both Ba-sensitive K conductance and H-K-ATPase

mediate the effect of sodium removal; 2) sodium competes with K for transport via the H-K-ATPase.

CHAPTER 1
INTRODUCTION

1.1 Background

Potassium (K) is one of the most abundant cations in the body and plays an important role in a variety of physiological functions. Cell growth, division, volume regulation, and acid-base balance depend on a high intracellular K activity. Reduction in K intake induces hypertension and supplement of K intake ameliorates hypertensive symptoms. High potassium concentration in cells and low concentration in the extracellular fluid contribute to the electrical properties of both excitable and nonexcitable tissures. Changes in either intracellular or extracellular K activity result in modification of both intracellular and extracellular pH. Potassium affects muscle metabolism, in broad term, by four different ways: by regulation of muscle blood flow during exercise; by its role in carbohydrate metabolism; by its effect on insulin secretion; and by its direct effect on muscle itself.

The performance of these vital functions by potassium is dependent on a variety of regulatory mechanisms that maintain K homeostasis. Figure 1-1 (Giebisch et al., 1981) schematically depicts the distribution of potassium ion in the body. As can be seen, most of potassium is located within cells, especially within muscle cells, with smaller amount in liver and erythrocytes. Potassium is the most abundant intracellular cation. Only 2% of body's potassium is distributed in the extracellular compartment, normally at

concentrations varying between 3.5 to 5 mM. Therefore, the loss or gain of an amount of potassium equivalent to 1% of total body potassium from the extracellular fluid may halve or double the potassium in plasma and the concentration ratio of potassium across the plasma membrane. This could substantially affect the electrical polarization of both excitable and nonexcitable tissue and be potentially lethal. In contrast, translocation of similar amount of potassium into or from the intracellular compartment only has a minimal effect on the concentrations. Therefore, the mechanisms controlling extracellular K must be more sensitive than those responsible for control of the intracellular potassium content. In fact, because of the highly regulated control mechanisms, potassium intake can increase over 20-fold with little change in body potassium content. Maintenance of this homeostasis reguires a balance between intake and excretion, so-called external potassium balance. The main route for potassium intake is the intestinal absorption which is not subject to specific control mechanisms. There are, however, three mechanisms that maintain the level of extracellular potassium within a critically narrow range.

1. When potassium intake increases, potassium can be translocated from the extracellular compartment to the intracellular fluid so that only a small fraction of any potassium added remains in the extracellular fluid. Conversely, extracellular potassium losses can be alleviated by shifting this ion from the cellular pool to the extracellular fluid. This distribution of potassium between extracellular and intracellular fluids, so-called internal potassium balance, is regulated by a variety of hormones such as aldosterone, catecholamines and insulin and by acid-base balance.

2. The colon has a capacity for potassium secretion and absorption which may be stimulated when the kidney's ability to handle potassium balance is compromised.

3. The kidney plays the key role and is ultimately for the appropriate response to changes in potassium excretion to match potassium intake. As shown in Figure 1-2 (Giebisch et al., 1981), in an individual ingesting 100 mmol of K per day, the proximal tubules absorbs up to 70% of filtered potassium which is approximately 600 mmol per minute. The Loop of Henle absorbs additional 22% of filtered K. When the luminal fluid deliveries to the early of distal tubule, only less than 10% of filtered K, approximately 50 mM, are left. Potassium secretion commences in the initial collecting duct and continues along the cortical collecting duct. The collecting duct (CD) is the final segment to determine net K excretion (Giebisch et al., 1981; Wright and Giebisch, 1992). In this case, the collecting duct transports net 50 mmol K into the urine. Therefore, 100 mmol potassium appears in the final urine, and the balance between potassium intake and output is maintained.

1) Proximal tubule. Our present state of knowledge about the role of the proximal tubule in the regulation of potassium excretion is largely derived from *in vivo* free-flow micropuncture of tubules accessible at the kidney surface (Giebisch et al., 1981). This segment absorbs the largest amount of potassium, which is mediated by both active and passive processes. However, the exact mechanisms explaining K absorption remain to be elucidated. The K concentration in samples of fluid from the proximal tubule has been found to be close to the concentration in plasma, suggesting that the rate of reabsorption of K along the proximal convoluted tubule is rather rigidly coupled to sodium

and water absorption. A direct comparison of extent of fluid absorption with the range of potassium concentration in the collected fluid samples has indicated that under normal condition, similar fractions of water, sodium, and potassium are reabsorbed between the glomerulus and the last accessible part of the proximal tubules (Beck et al., 1973; Diezi et al., 1976).

2) Loop of Henle. Approximately 5 to 15 % of the amount of potassium filtered reaches the early distal tubule of superficial nephron, and that this amount of potassium is not subject to a significant changes in spite of that the amount of K in the final urine may be changed as much as 200-fold (Malnic et al., 1964; Malnic et al., 1971).

3) Collecting duct. A great deal of evidence has demonstrated that K excretion is not dependent on K filtration but rather dependent on K secretion (blood-to-lumen) (Wright, 1987). The recent studies have suggested that K excretion is also dependent on K reabsorption (lumen-to-blood) by the collecting duct (Mujais and Katz, 1992). Because this segment is the final tuning site for potassium excretion, the amount of K that appeares in the urine is determined by net balance between potassium secretion and absorption processes which are critically regulated according to the different needs of the body. High K intake stimulates K secretion, whereas low K intake increases K absorption by this segment (Mujais and Katz, 1992; Wingo, 1987; Wright and Giebisch 1992).

1.2 Potassium Secretion by the Cortical Collecting Duct

Berliner and Kennedy (1948) and Mudge et al. (1948) were the first to dissociate K excretion from K filtration from their clearance studies. They reported that kidneys regularly responded to certain experimental manipulations by excreting K at the rate exceeding the rate of K filtration, suggesting that

potassium secretion is an important factor determining potassium excretion. Subsequently, Davidson and his associates (1958) performed another elegant studies in order to gain insight into the mechanisms of K secretion. They observed that when the glomerular filtration rate was reduced by about 30%, sodium excretion decreased by 80%, and potassium excretion decreased by 50%. In contrast, if the rate of sodium excretion was maintained at a high level by infusing salt and administering diuretics, then similar reduction in the glomerular filtration rate did not reduce potassium excretion. These data were taken as a clear evidence that potassium excretion is independent of potasium filtration and that potassium secretion is related to the amount of Na delivery. Since the early work of Grantham et al. (1970), the cortical collecting duct has been firmly established as the main site of potassium secretion. During past several decades, tremendous efforts have been devoted to understand K secretory mechanisms, and this process has been relatively well characterized.

It has been believed that at least four systemic factors affect primarily potassium secretion: potassium intake and total body potassium content; acid-base balance; sodium intake and extracellular fluid volume; chloride and nonchloride anions delivery (Wright, 1987). Generally speaking, the observations that increases in potassium intake and in total body potassium content stimulate potassium secretion have been attributable to the increase plasma K and circulating level of aldosterone. Changes in acid-base balance affects potassium excretion mainly because they modify distal flow rate, intracellular and tubular pH, and the anion composition of luminal fluid of distal tubule. Increases in sodium intake and in extracellular fluid volume promote potassium secretion (although they supress plasma aldosterone concentration) mainly by increasing

the driving force for K secretion and the flow rate of fluid passing the cortical collecting duct. Reduction of chloride level in the distal luminal fluid stimulates potassium secretion by stimulating potassium and chloride co-transport mechanism. Elevated concentrations of nonchloride anions in arterial plasma generally increase potassium secretion through modification of the anion composition of distal tubule fluid. Because of the relevance to the present studies, this section will be limited to review the relationship of acid-base disturbances and sodium absorption with potassium secretion and the current knowledge of the cellular model underlying potassium secretion.

Perhaps, Toussaint and Vereerstraeten (1962) first examined the relationship between acid-base balance and potassium secretion using clearance methods. The results are shown in Figure 1-3, in which the rate of renal potassium excretion is plotted as a function of plasma potassium concentrations at three different pH levels. It is clear that metabolic alkalosis stimulates and acute metabolic acidosis depresses the urinary excretion of potassium. The site of the alteration of potassium secretion during acid-base disturbances has been investigated more definitively by several approaches including free-flow micropuncture (Malnic, 1971) and *in vitro* microperfusion (Boundry et al., 1976). It is known that infusion of bicarbonate, or administration of carbonic anhydrase inhibitor, or hyperventilation of animals stimulates K secretion along the distal convoluted tubule (Kubota et al., 1983). In contrast, both acute metabolic and respiratory acidosis decreases potassium secretion in the distal tubule (Giebisch and Stanton, 1979; Malnic et al., 1971). Boundry et al. (1976) have demonstrated that potassium secretion by the cortical collecting duct was reduced when perfused *in vitro* by lowing luminal pH. The development of

electrophysiology techniques, especially the patching-clamp techniques, has extended our understanding of mechanisms explaining the relationship between acid-base disturbance and K secretion at the cellular level. Potassium secretion involves the translocation of this ion across the double-membrane. First, potassium is actively uptaken to cell by Na-K-ATPase located in the basolateral membrane. Second, potassium passively exits out of the cell to lumen in part through K-conductive pathway. Potassium conductance is pH sensitive. Decrease in pH has been shown to inhibit this conductive pathway (Stanton et al., 1982; O'Neil, 1983). Recently, low-conductance K channels have been identified in the apical membrane of the cortical collecting duct (Frindt and Palmer, 1989; Wang et al., 1990). The characteristics of this type of channels have made it to be a strong candidate for potassium conductance. Reducing pH also decreases open probability of this type of channels (Wang et al., 1990). Acidosis has been shown to inhibit Na-K-ATPase and reduce intracellular potassium activity (Giebisch, 1987). Inhibition of Na-K-ATPase and reduction of intracellular potassium activity inhibit K secretion by reducing the driving force for K exit out of the apical membrane (Giebisch, 1987; Wingo, 1984; Wright and Giebisch, 1992). Figure 1-4 summarizes the effect of different types of acid-base disturbances on potassium secretion obtained from micropuncture studies in the rat.

The idea that potassium secretion was coupled to sodium absorption was proposed on the basis of three major observations made in the late of fifties and the early of sixties. (1) In 1958, Davidson et al. (1958) reported their observations that sodium delivery drastically stimulated potassium secretion, particularly when sodium excretion was initially maintained at low level; (2)

Adrenal steroids increased both sodium absorption as well as potassium secretion (Sansom and O'Neil, 1985 & 1986); and (3) there was no evidence suggesting that tubular anion secretion could account for the amount of potassium secreted. In the *in vitro* perfused tubules, the experimental minipulations expected to abolish Na absorption such as removal of luminal Na, blocking Na entry by amiloride, and inhibiting basolateral Na exit by ouabain decreased K secretory flux (Stokes, 1981; Wingo, 1984). These studies not only reproduced the *in vivo* observations, but also provided the clear evidence of dependence of potassium secretion on sodium transport. The advance of technology has permitted us to understand the effect of sodium on potassium secretion at the cellular level. In all of the epithelial cells, sodium movement across the apical membrane are passive and driven by sodium concentration gradient and electrical potential gradient. The sodium concentration gradient is maintained by Na-K-ATPase located at the basolateral membrane. Sodium entry to the cortical collecting duct from the apical membrane is not exceptional. This sodium permeability is stimulated by circulating level of aldosterone (Sansom and O'Neil, 1985) and inhibited by administration of amiloride, a potassium-sparing diuretics (O'Neil and Boulpaep, 1979). The prevailing explainations for dependence of potassium secretion on sodium are listed as following (Wright and Giebisch, 1992): first, Na entry to the cell depolarizes apical membrane pontential, the depolarization of apical membrane potential generates a diffusion potential that favors K secretion; second, Na-K-ATPase is essential to maintain intracellular K activity above its electrochemical equilibrium, which is critical for potassium secretion, sodium entry into cell would provide the appropriate stimulus for increased activity of the basolateral Na-K-ATPase.

Two major cell types have been identified in the CCD (Figure 1-5). The majority cell type is the principal cell which is believed to be responsible for K secretion (Wright and Giebisch, 1992). The minority cell is the intercalated cell. More and more evidence suggests that this type of cell participates in proton secretion and potassium absorption (Mujais and Katz, 1992). Current concepts of the principal cell mode are based on the double-membrane model originally proposed by Koefoed-Johnsen and Ussing (1958). Its essential features and some modifications are summarized in Figure 1-6. (1) The apical membrane of cell is highly and selectively permeable to Na and K; (2) the basolateral cell membrane is highly potassium selective, (3) the only active transport operation is the ATP-driven sodium-potassium exchange pump in the basolateral cell membrane. A still growing fund of information derived from the efforts of many investigators, especially Drs. Berliner and Giebisch, and their coworkers, during past several decades has significantly contributed to the conversion of this " simple " original model into the more complex cell model that endows the tubular cells with capacity of potassium secretion. The current knowledge of principal cell with respect to K secretion is summarized in Figure 1-5.

1. Sodium (Na) enters the cell from luminal fluid passively through amiloride-sensitive Na conductive pathway. The sodium permeability plays an important role in potassium secretion as discussed before.

2. A Ba-sensitive K conductance is present in the apical membrane. This highly K-selective pathway provides an important route for potassium secretion from cell to lumen. The K-conductance accounts for the majority of conductance in the apical membrane of the principal cell, and is stimulated after administration of mineralocorticoids or increase in K intake, and inhibited

by acidification or by lowing K intake. This transport step has been extensively demonstrated to be passive in nature. Accordingly, lowing the potassium concentration in the lumen or increasing lumen-negative potential through external current application stimulates potassium secretion by steeping the electrochemical potential difference between cytoplasma and luminal fluid.

3. Evidence based on *in vitro* microperfusion study is consistant with potassium secretion in part through K-Cl cotransport (Ellison et al, 1985; Wingo, 1989). Reduction of chloride concentration in the perfusate stimulates potassium secretion. This component of potassium secretion is not associated with alterations in the transepithelial voltage and, importantly, is not blocked by barium.

4. Na-K-ATPase located in the basolateral membrane is the main transport element. This active uptake step responds to changes in acid-base disturbances, changes in plasma potassium level, and alterations in circulating level of plasma aldosterone. The increase in the plasma level of K or aldosterone stimulates Na-K-ATPase activity, and acidosis inhibits the activity of this enzyme.

1.3 Potassium Absorption by the Cortical Collecting Duct

In contrast to potassium secretion, little is known about the mechanisms of K absorption. As early as 1954, Spargo (1954) reported that the first mophologic changes during K depletion were in the collecting duct with a hyperplastic lesion developing in the inner red medulla, suggesting that collecting duct might participate in K conservation. Ten years later, from the micropuncture studies, Malnic, Klose and Giebisch (1964) demonstrated that during K depletion urinary fractional excretion of K was less than fractional

delivery of K to the late of distal nephron of superficial nephrons. This is the first physiological evidence of involvement of the collecting duct in active K absorption in response to K depletion. The postulate for active potassium absorption was based on the conditions which were characterized by complete absence of net potassium secretion, yet persistence of a distinctly lumen-negative transepithelial voltage. In the absence of other transport mechanisms, potassium should be secreted to lumen down its electrochemical gradient. To counterbalance the potassium secretion, an active force must move potassium out of the lumen. This suggests that an active potassium absorptive mechanism is present in the apical cell membrane, and the segment traditionally conceived as K-secretory also plays K conserving roles, especially when the balance of K requires it. Linas et al. (1979) studied the adaptation of the rat to a low K diet and found that the recovery of distally injected ^{42}K was significantly reduced 72 hours after exposure to the diet. Wingo (1987) has shown that K absorption was increased by the *in vitro* perfused outer medullary collecting duct dissected from K-restricted rabbits and provided the direct evidence substantiating the role of the collecting duct in K conservation. The further observations from mophological studies have demonstrated that the intercalated cells of the collecting duct undergo hypertrophy and ultrastructural changes during K restriction and acidosis, suggesting that these cells are involved in K absorption and H secretion (Hansen et al., 1980; Ordonez and Spargo, 1976; Stanton et al., 1981). However, the mechanisms underlying these observations remain unclear. The discovery of K-ATPase in rat and rabbit kidney made by Doucet and Marsy in 1987 (1987) has sheded more light on this poorly understood component. ATP hydrolysis by this enzyme is K-dependent and

reaches the maximal activity with 1 mM K (K_m about 0.2-0.4 mM). Sodium, choline, chloride and sulfate do not stimulate ATP hydrolysis by this enzyme. Ouabain, a specific inhibitor of Na-K-ATPase does not inhibit the activity of this enzyme, indicating that this enzyme is different from Na-K-ATPase. In contrast, omeprazole, an inhibitor of gastric H-K-ATPase, inhibits ATP hydrolysis by this enzyme, suggesting that this enzyme is pharmacologically similar to gastric H-K-ATPase. Because vanadate also inhibits this enzyme, K-ATPase has been classified as E_1-E_2 type ATPase. The activity of this enzyme is proportional to the density of intercalated cells, highest in the connecting duct, intermediate in the cortical collecting duct, and lowest in the outer medullary collecting duct, and not detectable in all other nephron segments. Hydrolysis of ATP by this enzyme in rat kidney increased by 100-200% following adaptation of the animals to a low K diet. In 1988, Garg and Narang (1988) reported a similar K-ATPase was also present in the rabbit collecting duct from their fluorometric microassay studies. This enzyme shares similar distribution pattern in the kidney as to that reported By Doucet and Marsy. This enzyme is inhibited not only by omeprazole and vanadate, but also by SCH28080, an imidazopyridine derivative shown in Fig. 1-7 which has been extensively studied in the gastric system and demonstrated to be a specific inhibitor of gastric H-K-ATPase (Beil et al., 1986 & 1987 & 1988; Dantzig et al., 1991; Mendlein and Sachs, 1990; Scott et al., 1987). Chronic hypokalemia stimulates the activity of this enzyme, and chronic hyperkalemia suppresses this enzyme (Garg and Narang, 1989). In 1989, Wingo (1989) observed that omeprazole inhibited both net K absorptive flux and total CO_2 flux in the in vitro microperfused medullary collecting duct without significantly changing the transepithelial

voltage, providing the functional evidence that an H-K-ATPase is present in this segment. The results from microenzymatic assay indicate that K-ATPase is stimulated by K and by Rb in a similar fashion. The apparent stoichiometry of this enzyme is 1 Rb:1 ATP. Adaptation of animals to a low K diet not only increases ATP hydrolysis, but also Rb uptake by this enzyme. SCH28080 has been demonstrated to inhibit initial Rb uptake in the rat collecting duct (Cheval et al., 1991) and proton (H) secretion in the amphibian nephron (Planelles et al., 1991). Although at the molecular level whether renal H-K-ATPase is similar to gastric H-K-ATPase, or colonic H-K-ATPase has not been reconciled, several investigators have expressed mRNA of a putative H-K-ATPase in the kidneys (Gifford et al., 1991; Okusa et al., 1990), and this expression is enhanced by respiratory acidosis. By using mouse monoclonal antibodies against hog gastric H-K-ATPase, Wingo et al. (1991) revealed diffuse cytoplasmic staining indicating H-K-ATPase immunoreactivity in intercalated cells in the cortical collecting duct and outer medullary collecting duct of both rat and rabbit. The percentage of H-K-ATPase immunoreactive cells has been demonstrated to correspond to the percentage of intercalated cell except in rat CCD in which the percentage of positive staining is less than the percentage of intercalated cells.

Besides it has been well characterized in the gastric gland, a similar H-K-ATPase has been also identified in colonic epithelium (Kaunitz and Sachs, 1986; Suzuki and Kaneko, 1987 & 1989) and turtle urinary bladder (Sharma et al., 1991). The evidence obtained from functional studies suggests that H-K-ATPase may be also present in the amphibian jejunum (Imon and White, 1984), smooth muscle cell and the *Manduca sexta* embryonic cell line CHE (English and Kantley, 1985).

1.4 The Aims and Objectives of the Present Studies

The discovery of H-K-ATPase is a novel approach to characterize K absorption. Its significance in both understanding basic K transport mechanism and clinical implications may significantly extend our present knowledge. This dissertation attempts to characterize potassium permeation by the CCD with emphasis of H-K-ATPase in the conditions which are close to *in vivo* without equivocating the interpretation of the results. *In vitro* micropersion technique is able to serve this dual-purposes. Potassium permeation was evaluated as ^{86}Rb lumen-to-bath efflux coefficient (K_{Rb} or Rb efflux). The H-K-ATPase activity was assessed as SCH28080-sensitive K_{Rb} or SCH28080-sensitive Rb efflux. The specific objectives are summarized as following:

1. Because H-K-ATPase secretes proton in exchange of K absorption, we will determine whether acute peritubular acidosis (10% CO_2) stimulates Rb efflux, whether this stimulation is dependent on the presence of functional H-K-ATPase. We will also identify the intracellular and basolateral mechanisms explaining the effect of 10% CO_2 on Rb efflux.

2. Because luminal amiloride and peritubular ouabain has been shown to inhibit net K excretion, we will examine whether luminal amiloride and peritubular ouabain increases Rb efflux, and whether the effects of amiloride and ouabain are mediated by K-conductance or by H-K-ATPase. Because of the indirect evidence suggesting that an apical Na-K-ATPase may participate in potassium conservation, we will identifiy the roles of H-K-ATPase and Na-K-ATPase in mediation of Rb efflux.

3. Because removal of luminal Na produces similar effect on ion transport in many cases as the addition of luminal amiloride, we will examine

whether Na removal stimulates Rb efflux, whether the effect of Na removal is mediated by K-conductance or by H-K-ATPase or both. We will also determine the mechanism underlying the effect of Na removal on H-K-ATPase.

Additional efforts have been made to study hormonal regulation of this pump. The results of metabolic studies on a low Na, low K diet, and a low K diet are also reported[1].

[1] All of the studies were funded by the Department of Veterans Affairs

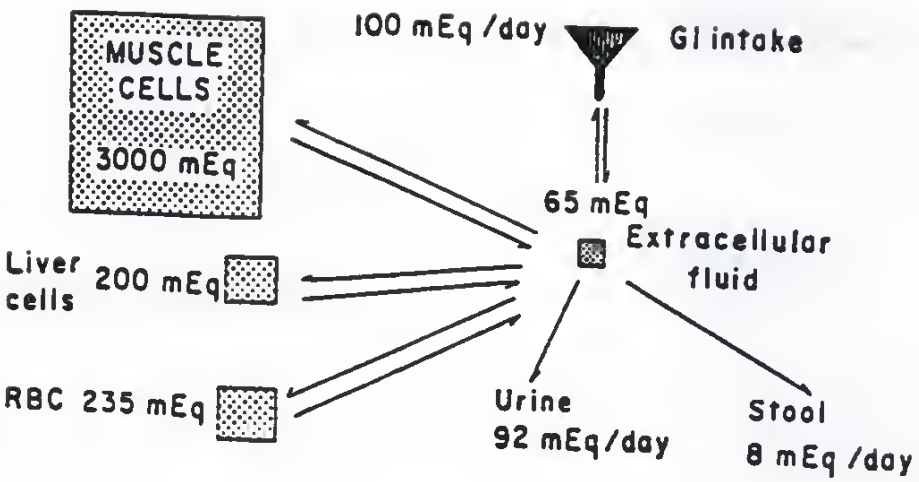


Figure 1-1. Distribution of potassium in the body, including routes of acquisition and excretion (Giebisch et al, 1981).

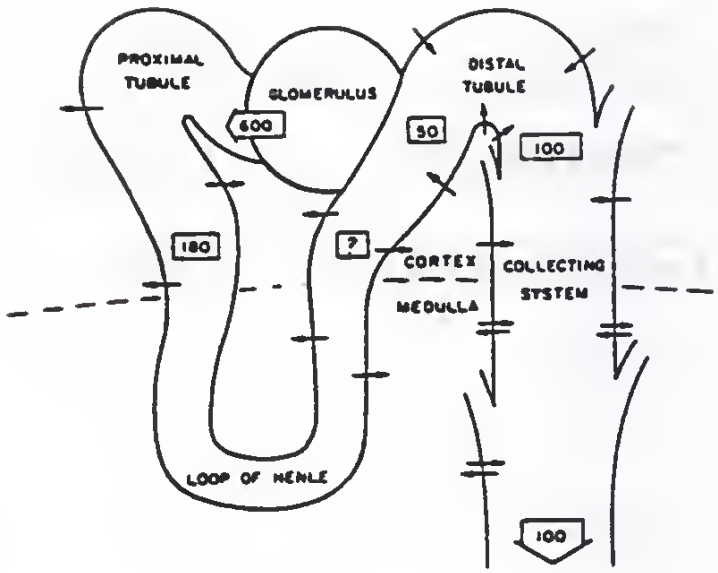


Figure 1-2. Segmental analysis of tubule potassium transport. Arrows indicate the direction of net transtubular movement (Giebisch et al., 1981).

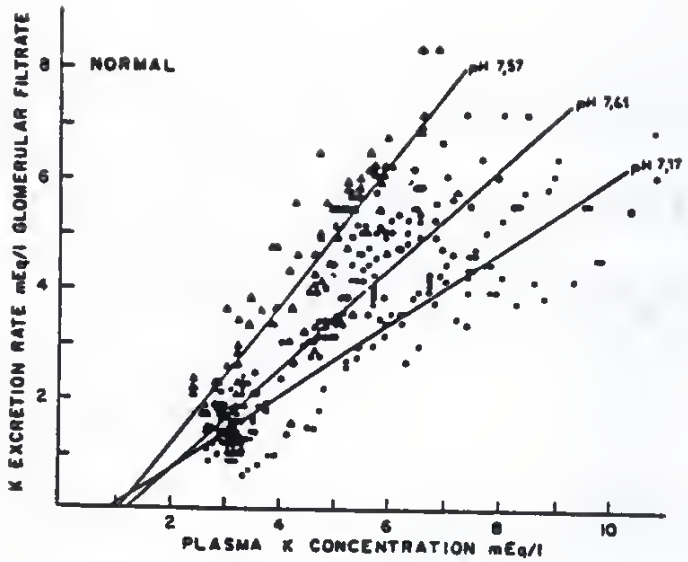


Figure 1-3. Urinary potassium excretion as a function of plasma potassium concentration in control animals and in alkalotic and acidotic conditions (Toussaint and Vereerstraeten, 1962).

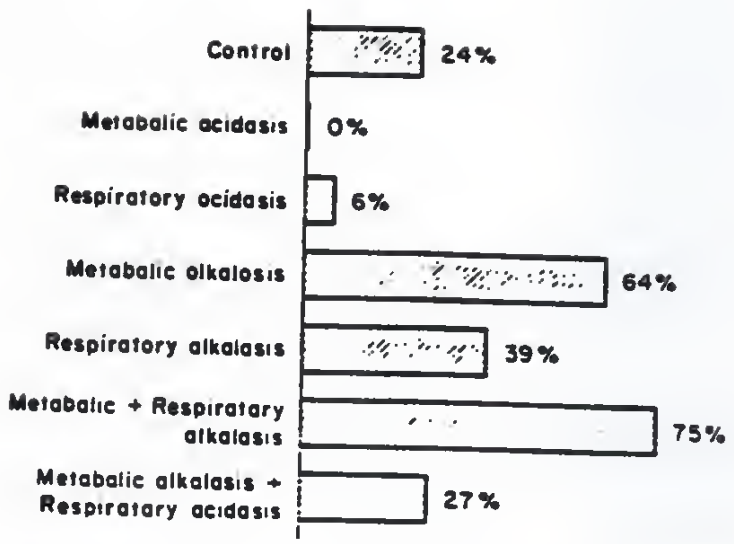


Figure 1-4. Effect of different types of acid-base disturbances on potassium secretion by distal tubule (Giebisch et al, 1981).

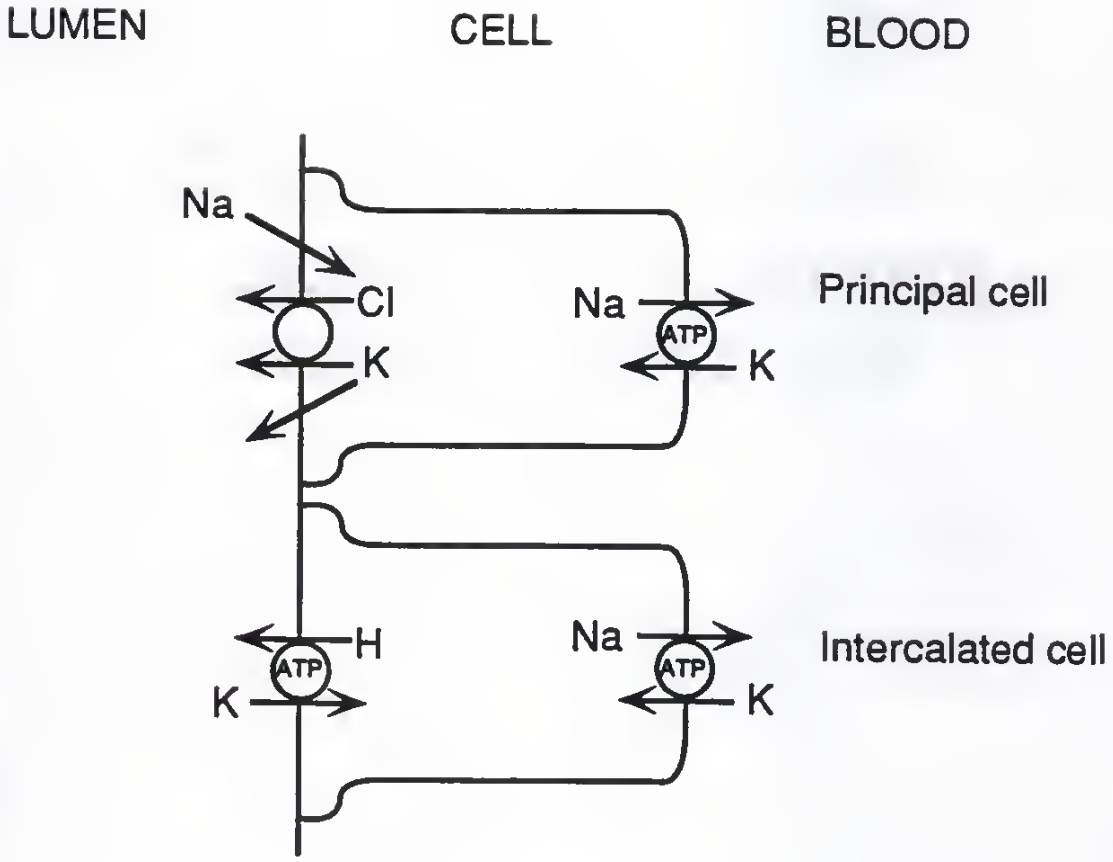


Figure 1-5. The cellular models of the principal cell (top) and the intercalated cell (bottom).

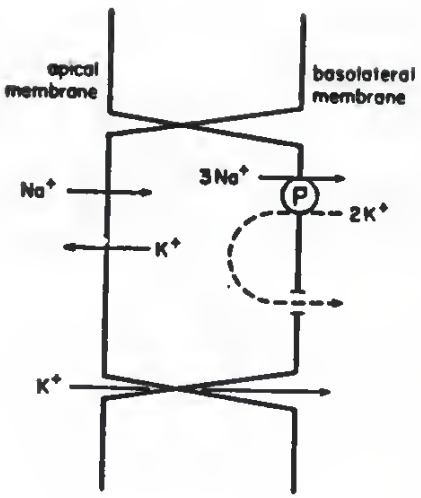
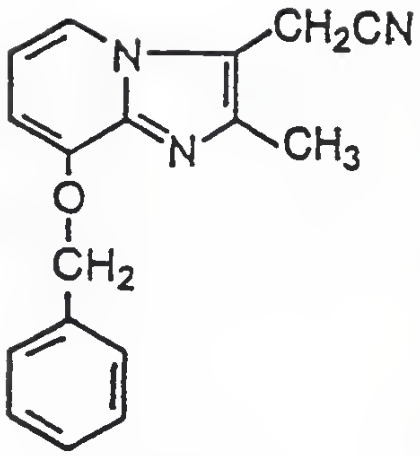


Figure 1-6. Cell model of epithelial sodium and potassium transport (Giebisch, 1987).



SCH 28080

Figure 1-7. The chemical structure of SCH28080.

CHAPTER 2
GENERAL METHODOLOGY

2.1 *In Vitro* Microperfusion

In vivo conditions. Female New Zealand White rabbits were maintained on a regular diet (Ralston Purina), or a low K (K-restricted) diet (0.25% K, TD 87433, Teklad, Madison, WI) as appropriate and allowed free access to tap water. The exposure time to the low K diet was at least for 4 days before experimentation. The majority of experiments were performed on the K-restricted rabbits unless indicated. Rabbits were adapted to the K-restricted diet in order to enhance the signals.

In vitro methods. Standard in vitro microperfusion methods (Burg et al, 1966) as modified in this laboratory (Wingo, 1984 & 1985) were used. Briefly, rabbits were decapitated, the left kidney was quickly removed, and 1- to 2-mm slices were placed in a chilled petri dish containing an artificial ultrafiltrate of plasma. Dissection proceeded superficially from the corticomedullary junction. Tubules were transferred to a thermostatically controlled chamber (37°C), and the two ends of the tubule were aspirated into holding pipettes (Figure 2-1). The perfusing pipette was advanced 100 μm beyond the holding pipette, and transepithelial voltage (V_T) was continuously monitored by means of Ag/AgCl electrodes and a FD-223 high-impedence electrometer (World Precision Instruments, Sarasota, FL). The bath solution was continuously exchanged at the rate of 0.64 ml·min^{-1}. The perfusate contained 50 μCi of [methoxy-^{3}H]inulin

exhaustively dialyzed according to the method of Schafer et al. (1974). The equilibration time between two periods was 30 minutes unless indicated. Effluent fluid was collected into a constant-volume pipette for measurement of volume flux, isotopic flux and net chemical flux. Volume flux was determined from timed collections of the effluent fluid using the equation $Jv = ([cpm_o/cpm_i-1]\cdot V_o)/L$, where Jv is the net volume absorption in nanoliters per millimeter per minute, cpm_o and cpm_i are the [^{3}H]-inulin counts per minute per nanoliter in the collected and the perfused fluid, respectively, V_o is the rate of fluid collection in nanoliters per minute, and L is the tubule length in millimeters. In all experiments, the percent of [^{3}H]-inulin leak was less than 5% or the experiment was discarded. In most tubules the leak rate was less than 2%. Isotopic ^{86}Rb was used because it has been shown to be a qualitative marker of K efflux (134), and is transported by the renal H-K-ATPase quantitatively similar to K (14). The ^{86}Rb lumen-to-bath efflux (K_{Rb}) was determined by the disappearance of ^{86}Rb from the perfusate according to the following equation:

$$K_{Rb} = \frac{V_o}{L} \cdot 2\frac{\overset{*}{Rb}_i - \overset{*}{Rb}_o}{\overset{*}{Rb}_i + \overset{*}{Rb}_o}$$

where Rb_i and Rb_o are the ^{86}Rb counts per minute per nanoliter in the perfused and collected fluid, respectively. The Na efflux rate coefficient (K_{Na}) was calculated by a similar equation. Counts for ^{3}H, ^{86}Rb and ^{22}Na were measured by a liquid scintillation counter (LS-7800, Beckman Instruments, Irvine, CA). The overlap of ^{86}Rb or ^{22}Na counts in the ^{3}H channel was corrected as previously described (Zhou and Wingo, 1992). Net chemical flux was determined

by the equation $J_X = (V_o/L)\bullet([X]_i-[X]_o)$ where V_o and L are as before and $[X]_i$ and $[X]_o$ are the sodium or potassium concentration of the perfusate and collectate, respectively. At least three and generally four collections were obtained for measurement of flux. The passive paracellular K flux was calculated according to the Goldman flux equation

$$J_K^P = \frac{zF}{RT} \cdot V_T \cdot P_K \frac{[K]_b - [K]_l \exp[V_T zF/(RT)]}{1 - \exp[V_T zF/(RT)]}$$

where P_K is the paracellular K permeability, $[K]_b$ is the bath K concentration, $[K]_l$ the mean luminal K concentration, and z, F, R, and T have their usual meaning. All chemicals were analytical grade or the highest available purity. ^3H inulin, ^{86}Rb and ^{22}Na were obtained from New England Nuclear (Boston, MA).

2.2 Flameless atomic absorption spectrophotometry

Flameless atomic absorption spectrophotometry described by Wingo et al (1987) was used for the analysis of sodium and potassium concentrations in the perfusate and collectate in order to determine net sodium and potassium flux. Briefly, the standards and samples were placed on a clean silver platform, freshly polished, and immersed under 0.5 cm of water-equilibrated paraffin oil. The diluent was prepared from 100 μl Ultrex (J. T. Baker chemical Co.) and 100 ml ultra-high-purity (18 Mohm) water ("18 Mhom water," Continental Water System) in a clean polypropylene container. To prevent loss of the analyte, the samples were slowly dried before pyrolysis and atomization (951 AA/AE spectrophotometer and IL 655 furnance atomizer, Allied Analytic, Waltham, MA). Peak height integration started at the end of the pyrolysis and continues for 8 seconds in the single beam mode at a scale expansion of 1.00 for both

sodium and potassium. The first atomization step of 500 degree of centigrade to 1500 degree of centigrade in "0" second allows a rapid increase in temperature during atomization. The instrumental conditions for analysis of Na and K were set as followings : 589.6 and 766.5 nm, bandwidth; 0.15 and 1.0 mm, lamp current; 8.0 and 7.0 mamp. N_2 gas pressure was 12.0 psi. The spectrophotometer was allowed to warm up for at least one hour and then "auto zeroed" by atomizing without sample introduction into the furnance. The diluted samples were delivered into a pyrolytically coated graphite cuvette in the furnance with a 1- to 10-μl Eppendorf digital pipette. The exact volume chosen for a given analysis was determined by repetitive runs of the high and low standards and adjusting the delivery volume for an optimum absorption signal from the sodium and potassium channels. Each sample was analyzed for Na and K a minimum of three times; generally four to six runs were employed.

2.3 Statistical analyses

Data are expressed as mean ± standard error. Statistical analyses were performed by paried *t* test for the experiments containing two periods and by analysis of variance (ANOVA) for repeated measures of the experiments containing more than two periods. Post hoc comparisons were made by the Ryan-Einot-Gabriel-Welch *F* test. The null hypothesis was rejected at the 0.05 level of significance.

Figure 2-1. The cortical collecting duct perfused *in vitro*.

CHAPTER 3
EFFECT OF 10% CO_2 ON Rb EFFLUX

3.1 Introduction

Substantial evidence indicates that an H-K-ATPase is present at the apical membrane of the cortical collecting duct (Cheval et al., 1991; Doucet and Marsy, 1987; Garg and Narang, 1988; Gifford et al., 1991; Okusa et al., 1990; Planelles et al., 1991; Wingo, 1989; Wingo et al., 1989 & 1990; Wingo and Straub, 1991; Wingo and Zhou, 1990; Zhou and Wingo, 1992). This enzyme has been demonstrated to be responsible for potassium absorption in exchange of proton secretion, especially during K-restriction (Cheval et al., 1991; Wingo, 1989; Zhou and Wingo, 1992). Komatsu and Garg (1991) have shown that metabolic acidosis increases ATP hydrolysis by this enzyme, suggesting that acid-base balance regulates the activity of this enzyme. Perrone and McBride (1988) have demonstrated that 10% carbon dioxide (CO_2) increases rubidium absorption in colon, possibly mediated by a related pump. However, whether respiratory acidosis stimulates one of the physiologic functions of the renal H-K-ATPase, i.e. K absorptive flux, is unknown. Nevertheless, to our knowledge, the direct relationship between acidosis and K absorptive flux has not been demonstrated in the CCD. Therefore, the first objective of this study is to examine whether respiratory acidosis induced by 10% CO_2 increases K absorption, and whether this increment is dependent on the presence of a functional H-K-ATPase in the *in vitro* microperfused CCD from K-restricted rabbits.

The second issue is the dependence of proton secretion in the collecting duct on carbonic anhydrase. Carbonic anhydrase has been identified in the CCD (Dobyan and Bulger, 1982). Carbonic anhydrase catalyzes the hydration of CO_2 and an increase in pCO_2 results in a decrease intracellular pH (Cannon et al., 1985). The decrement in intracellular pH is a direct stimulus for H secretion (Cannon et al., 1985; Schwartz and Al-Awqati, 1985; Stetson and Steinmetz, 1983 & 1986; Van Adelsberg and Al-Awqati, 1986). Because H-K-ATPase participates in H secretion, it is plausible that the enhancement of K absorption resulted from the activation of H-K-ATPase by 10% CO_2 is carbonic anhydrase-dependent. However, several investigators have demonstrated that acidification by the collecting duct is in part independent of carbonic anhydrase (Cogan et al., 1979; DuBose and Lucci, 1983; Frommer et al., 1984; Laski, 1987; McKinney and Davidson, 1988). Sharma et al. (1991) have suggested that not all H-K-ATPase activity is dependent on the function of carbonic anhydrase. Therefore, the second objective of this study is to evaluate the role of carbonic anhydrase in the stimulation of H-K-ATPase-mediated K absorption and the role of carbonic anhydrase in maintaining basal rate of K absorptive flux in the K-restricted CCD.

The third issue is the intracellular mechanisms involved in the effect of 10% CO_2. Exocytotic insertion of H-K-ATPase in response to several stimuli has been demonstrated in gastric gland (Forte et al., 1981). This process is cytoskelton-dependent. The majority of exocytosis is also calcium (Ca)-dependent, more importantly, exocytotic fusion of H-ATPase has been shown to be Ca-mediated in the turtle urinary bladder (Schwartz and Al-Awqati, 1985 & 1986; Van Adelsberg and Al-Awqati, 1986). Therefore, the third objective of the

present studies is to evaluate the role of microtubules, intracellular calcium activity, and calmodulin in the stimulation of Rb efflux (presumably activation of H-K-ATPase) by 10% CO_2.

The fourth issue is the mechanism explaining basolateral K exit. In the rabbits adapted to a normal diet the minority cells (presumably intercalated cells) exhibit quite low basolateral K conductance (Muto et al., 1987). However, Schlatter and Schafer (1987) have identified that a Ba-sensitive K conductance is present in the basolateral membrane of both principal cells and intercalated cells of the CCD of rats. If a similar pathway is present in the K-restricted rabbits, this pathway could mediate K absorption. Therefore, the fourth objective of this study is to examine whether a Ba-sensitive pathway is present in the basolateral membrane of the CCD, and whether this pathway mediates the stimulation of K absorption by 10% CO_2 in the K-restricted rabbits.

The final issue to be addressed by the present studies is whether 10% CO_2 stimulates Rb efflux in the CCD dissected from normal rabbits.

3.2 Methods and Materials

The tubules was dissected in an artificial ultrafiltrate of plasma (in mM; Na 145; K 5; Cl 112; HCO_3 25; Ca 1.8; PO_4 2.3; Mg 1.0; SO_4 1.0; acetate 10; glucose 8; and alanine 5) gassed with 5% CO_2 containing additional 5% vol/vol fetal calf serum. The bath solutions were gassed with 5% CO_2 (pH = 7.4 ± 0.0) or 10% CO_2 (pH = 7.1 ± 0.0) as appropriate. The electrolyte and nonelectrolyte concentrations of the bath solution were identical to the dissection solution unless indicated. In the peritubular Ba study, the composition of bath were (in mM): Na 135; K 5; Cl 115.4; HCO_3 25; Ca 1.2; Mg 1.0; acetate 10; glucose 8; alanine 5; Ba 3; and mannitol 4.5. In the Ba

dose-response study, the composition of bath were (in mM): Na 135; K 5; Cl 106.4; HCO_3 25; Ca 1.2; Mg 1.0; PO_4 1.5; acetate 10; glucose 8; alanine 5; mannitol 26.5. The equilibration time between two periods was 30 minutes unless indicated. The perfusate was identical to bath except for the absence of fetal calf serum and gassed only with 5% CO_2 unless indicated. The composition of the perfusate for peritubular Ba study were (in mM): Na 138; K 5; Cl 109.4; HCO_3 25; Ca 1.2; Mg 1.0; acetate 10; glucose 8; alanine 5; mannitol 9; and PO_4 1.5. Because Ba precipitates with phosphate, HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid)-buffer was used as the perfusate in the Ba dose-response study. Warden et al () have shown that Rb efflux by the CCD perfused with HEPES-buffer is not significantly different from that perfused with bicarbonate-buffer. Total three perfusates listed in Table 3-1 were used in this set of studies.

SCH28080 (gift of Dr. James Kaninsky, Schering Corporation, Bloomfield, NJ) was dissolved in dimethyl sulfoxide (DMSO) and applied to the perfusate with final concentration of 10 μM. Methazolamide (American Cyanamide Company, Pearl River, NY), Bis-(2-amino-5-methyl-phenoxy)-ethane-N,N,N',N'-tetraacetic acid tetraacetoxymethyl ester (MAPTAM, Sigma, St. Louis, MO), and N-(6-aminohexyl)-5-chloro-1-naphthalene-sulfonamide (W-7, Sigma, St. Louis, MO) were also dissolved in DMSO and applied to bath with final concentration of 0.1 mM, 0.5μM, and 0.5μM, respectively. Colchicine (Sigma, St. Louis, MO) was dissolved in 0.9% NaCl solution and applied to bath with the final concentration of 0.5mM. The final concentration of 0.1 % DMSO was present in bath during basal period of methazolamide sets of experiments.

3.3 Results

To examine whether acute respiratory acidosis stimulates Rb efflux, we perfused the CCD with symmetrical Ringer's bicarbonate solution gassed with 5% CO_2 (5% CO_2 period) or 10% CO_2 (10% CO_2 period). In our initial studies we allowed one hour equilibration after obtaining basal flux rates prior to a second period of measurement in the presence of 10% CO_2. As shown in Figure 3-1, 10% CO_2 substantially increased K_{Rb} from 93.1 ± 23.8 nm·sec^{-1} to 249 ± 60.2 nm·sec^{-1} ($P < 0.05$, $n = 7$). Concomitantly the transepithelial voltage (V_T) became more lumen-positive from -2.4 ± 1.3 mV to -1.2 ± 0.9 mV ($P < 0.05$, $n = 7$, Fig. 3-1). The greater lumen-positive voltage could enhance paracellular Rb passive efflux. To examine whether the change in V_T accounted for the effect of 10% CO_2 on Rb efflux, we calculated the voltage-mediated Rb efflux by Goldman flux equation. As shown in Figure 3-2, the change in V_T can not explain for the increase in K_{Rb} by 10% CO_2. Voltage-mediated Rb efflux as predicted by Goldman flux equation was only 10.6 ± 3.7 nm·sec^{-1}, whereas the increment of 156 ± 58.4 nm·sec^{-1} was observed. To examine whether H-K-ATPase mediated the effect of 10% CO_2 on Rb efflux we repeated the experiments under the identical conditions except for the presence of luminal 10 μM SCH28080. Luminal SCH28080 totally abolished the stimulatory effect of 10% CO_2 on Rb efflux (76.4 ± 15.1 nm·sec^{-1} vs 76.8 ± 13.3 nm·sec^{-1}, $n = 5$, Fig. 3-3), although V_T became more lumen-positive during 10% CO_2 period (-14.6 ± 9.6 mV vs -10.7 ± 9.1 mV, $P < 0.01$, $n = 5$, Fig. 3-3). In additional experiments, there was no evidence to suggest that 0.1% DMSO affected either K_{Rb} or V_T (Table 3-2). These data suggest that an H-K-ATPase, not the change in V_T, mediates the enhancement of Rb efflux by 10% CO_2. To examine the time course of the stimulation of Rb efflux by 10%

CO_2, we perfused the CCD in the absence of luminal SCH28080. After measurement of the basal rate of Rb efflux (5% CO_2 period), effluent fluid was collected from 30 to 120 minutes after exposure to 10% CO_2. To evaluate the role of carbonic anhydrase in the stimulation of Rb efflux by 10% CO_2, 0.1 mM methazolamide was added to the bath from 120 to 180 minutes after exposure to 10% CO_2. We were not only able to reproduce the previous stimulation of K_{Rb} by 10% CO_2, but also observed this effect at the earliest collection time, i.e., 30 to 60 minutes later after the tubule exposure to 10% CO_2. This effect was persistent for the entire 10% CO_2 period. However, subsequent addition of methazolamide did not significantly inhibit K_{Rb} (72.4 ± 11.8 nm·sec^{-1}, 5% CO_2 period; 121 ± 29.9 nm·sec^{-1}, 135 ± 34.3 nm·sec^{-1}, and 133 ± 29.0 nm·sec^{-1}, 30-60 minutes, 60-90 minutes, 90-120 minutes after exposure to 10% CO_2, respectively; 133 ± 29.0 nm·sec^{-1}, methazolamide period, n = 6, Fig. 3-4). During the methazolamide period V_T was significantly more lumen-negative (-3.0 ± 2.2 mV, 5% CO_2 period; -2.4 ± 2.3 mV, -1.3 ± 1.8 mV, and -0.8 ± 1.6 mV, 30-60 minutes, 60-90 minutes, and 90-120 minutes after exposure to 10% CO_2, respectively; -7.6 ± 4.2 mV, methazolamide period, n = 6, Fig. 3-4). When the experiments were repeated under same conditions except for the presence of luminal 10μM SCH28080, SCH28080 totally blocked the enhancement of Rb efflux in response to 10% CO_2 and methazolamide had no significant effect on Rb efflux under these conditions (90.8 ± 16.5 nm·sec^{-1}, 5% CO_2 period; 80.5 ± 12.3 nm·sec^{-1}, 83.9 ± 14.3 nm·sec^{-1}, and 78.7 ± 10.9 nm·sec^{-1}, 30-60 minutes, 60-90 minutes, and 90-120 minutes after exposure to 10% CO_2, respectively; 81.9 ± 13.5 nm·sec^{-1}, methazolamide period, n = 7, Fig. 3-5), although V_T became more lumen-negative during methazolamide period

(-4.5 ± 3.1 mV 5% CO_2 period; -3.2 ± 2.5 mV, -2.0 ± 2.8 mV, and -0.8 ± 2.6 mV, 30-60 minutes, 60-90 minutes, and 90-120 minutes after exposure to 10% CO_2, respectively; -7.8 ± 3.2 mV, methazolamide, n = 7, Fig. 3-5). These data not only confirm that an H-K-ATPase mediates the stimulatory effect of 10% CO_2 on K_{Rb}, but also suggest that an existing H-K-ATPase mediates this effect. Again, the parallel V_T responses to 10% CO_2 regardless of the presence or absence of SCH28080 indicate that the changes in V_T do not explain the effect of 10% CO_2 on Rb efflux. The lack of inhibitory effect of methazolamide on K_{Rb} by subsequent addition after 10% CO_2 implies that carbonic anhydrase may not be necessary to maintain the activation of H-K-ATPase by 10% CO_2. In the presence of luminal SCH28080, methazolamide did not reduce Rb efflux either, suggesting that methazolamide had no effect on the other pathways of K permeation. To examine whether carbonic anhydrase is required for initiating the stimulation of Rb efflux by 10% CO_2, we simultaneously exposed the CCD to methazolamide and 10% CO_2 after measurement of the basal rate of Rb efflux. Under these conditions we were not able to detect the stimulatory effect of 10% CO_2 on Rb efflux (98.6 ± 14.1 $nm \cdot sec^{-1}$ vs 86.2 ± 16.5 $nm \cdot sec^{-1}$, n = 6, Fig. 3-6), although methazolamide significantly made V_T more lumen-negative from 1.3 ± 0.9 mV to -4.1 ± 1.2 mV ($P < 0.005$, n = 6, Fig. 3-6). Time-control experiments demonstrated that perfusion time did not significantly affect K_{Rb} or V_T (Table 3-3). These data suggest that carbonic anhydrase is necessary for initiating the stimulatory effect of 10% CO_2 on Rb efflux. To examine whether methazolamide inhibits the basal rate of Rb efflux, we perfused the CCD in the presence of 5% CO_2 throughout the experiments instead of changing to 10% CO_2. As shown in Figure 3-7, methazolamide did

not significantly inhibit K_{Rb} (60.4 ± 10.1 nm·sec^{-1} vs 59.1 ± 10.6 nm·sec^{-1}, n = 6), although the effect of methazolamide on V_T was reproducible (-12.9 ± 2.7 mV vs -27.1 ± 4.8 mV, $P < 0.05$, Fig. 3-7, n = 6). This suggests that the basal Rb efflux may be dependent on methazolamide-insensitive pathway.

Exocytotic insertion of H-K-ATPase in response to several stimuli has been demonstrated in gastric gland (Forte et al, 1981). This process is cytoskelton-dependent. To examine whether microtubules mediate the effect of 10% CO_2 on Rb efflux, the tubules were simultaneously exposed to 10% CO_2 and 0.5 mM colchicine, an inhibitor of tubulin polymerization. Colchicine totally blocked the stimulatory effect of 10% CO_2 on Rb efflux (85.0 ± 15.0 nm·sec^{-1} vs 81.3 ± 13.6 nm·sec^{-1}, n=6, Fig. 3-8), indicating that the stimulation of Rb efflux is dependent on intact microtubule function. Meanwhile, colchicine had no significant effect on V_T (-12.9 ± 4.9 mV vs -15.6 ± 5.0 mV, n=6, Table 3-4). To further test the insertion hypothesis, we examined the effect of colchicine after stimulation of K_{Rb} by 10% CO_2. Under these conditions, colchicine had no inhibitory effect on Rb efflux (72.5 ± 7.9 nm·sec^{-1}, 5% CO_2 period; 97.3 ± 10.1 nm·sec^{-1}, 10% CO_2 period*; vs 110 ± 13.2 nm·sec^{-1}, 10% CO_2 plus colchicine period*, *$p<0.05$ compared with 5% CO_2 period, no significant difference between 10% CO_2 and 10% CO_2 plus colchicine period, n=6, Fig. 3-8). Voltage was significantly altered by this maneuver (-11.2 ± 7.1 mV, 5% CO_2 period; -16.9 ± 9.4 mV, 10% CO_2 period*; -17.4 ± 9.5 mV, 10% CO_2 plus colchicine period*, * $p<0.05$ as compared with 5% CO_2 period, n=6, Fig. 3-3). These data are consistant with the postulation that exocytotic process mediates the effect of 10% CO_2 on Rb efflux.

Because the majority of exocytosis is Ca-dependent, more importantly, exocytotic fusion of H-ATPase has been shown to be Ca-mediated in the turtle urinary bladder (Schwartz and Al-Awqati, 1985 & 1986; Van Adelsberg and Al-Awqati, 1986), to examine whether the increase in intracellular Ca activity mediates the effect of 10% CO_2, the CCD was perfused in the presence of 0.5mM MAPTAM, an intracellular Ca buffer, throughout the experiments. In this case 10% CO_2 failed to stimulate Rb efflux (89.5 ± 19.5 nm·sec^{-1} vs 85.6 ± 23.6 nm·sec^{-1}, n=6, Fig 3-9). In addition, V_T became more lumen negative during 10% CO_2 period (-15.7 ± 5.9 mV vs -23.9 ± 6.8 mV*, *$p<0.05$, n=6, Table 3-5). To evaluate the role of calmodulin in the stimulation of K_{Rb} by 10% CO_2, the CCD was perfused in the presence of 0.5μM W-7, an antaganizer of calmodulin, throughout the experiments. In the presence of W-7, Rb efflux was significantly reduced (89.3 ± 14.0 nm·sec^{-1} vs 65.1 ± 13.0 nm·sec^{-1}, $p<0.05$, n=6, Fig. 3-9), suggesting that the stimulation of Rb efflux by 10% CO_2 is dependent on the functional calmodulin. Transepithelial voltage was not significantly altered (-12.2 ± 3.9 mV vs -14.4 ± 3.5 mV, Table 3-5).

Because Schlatter and Schafer (1987) have identified a Ba-sensitive K conductance located in the basolateral membrane of both principal cells and intercalated cells of the CCD of K replete rats, although the intercalated cells of the K replete rabbits exhibits a little Ba-sensitive K conductance (Muto et al., 1987). To examine whether a Ba-sensitive pathway is present in the K-restricted CCD of rabbits, and whether this pathway mediates the stimulation of Rb efflux by 10% CO_2, we perfused the CCD in the presence of 3mM Ba in bath. Pretreatment with Ba totally abolished the stimulation of K_{Rb} by 10% CO_2 (73.0 ± 8.2 nm·sec^{-1} vs 70.9 ± 8.3 nm·sec^{-1}, n = 6, Fig. 3-10) without

significantly affecting V_T (-2.2 ± 3.0 mV vs -0.0 ± 1.7 mV, n = 6, Fig. 3-10). This indicates that K conductance is present in the basolateral membrane under present conditions, and this conductive pathway mediates the stimulatory effect of 10% CO_2 on Rb effllux.

To test whether normal CCD has the similar response to 10% CO_2 as the K-restricted CCD, we examined the effect of 10% CO_2 on the CCD dissected from regular dietary rabbits. Because the apical Ba-sensitive K-conductance has been demonstrated to participate in Rb efflux (Warden et al., 1989; Wingo and Zhou, 1990) also because the decrease pH has been shown to inhibit this conductive pathway (Boudry et al., 1976; Wang et al., 1990), in order to eliminate the overshadowing effect of 10% CO_2 on K-conductance and H-K-ATPase, the tubules were perfused in the presence of luminal Ba. To find out what concentration of Ba maximally inhibits Rb efflux, the first set of experiments was designed to examine the dose-response of Ba with the perfusates of solution A, solution B, and solution C designated as 0 mM Ba, 2 mM Ba, and 4 mM Ba periods, respectively. The order of these three periods were rotated according to balanced Latin-square design (Cochran and Cox, 1957) to control for time-dependent effect on Rb efflux and the order of the periods did not significantly affect Rb efflux. As shown in Fig. 3-11, 2 mM Ba is sufficient enough to inhibit K_{Rb} (76.3 ± 14.6 nm•sec^{-1}, 0 mM Ba period; 32.9 ± 4.4 nm•sec^{-1}, 2 mM Ba period; and 32.1 ± 9.3 nm•sec^{-1}, 4 mM Ba period). The next set of experiments was performed in the presence of luminal 2 mM Ba to examine the effects of 10% CO_2 and 0.1 mM methazolamide. 10% CO_2 and methazolamide had no significant effect on Rb efflux (48.1 ± 7.2 nm•sec^{-1}, 5% CO_2 period; 42.5 ± 8.3 nm•sec^{-1}, exposure to 10% CO_2 from 30 to 60

minutes; 44.6 ± 7.5 nm•sec^{-1}, exposure to 10% CO_2 from 60 to 90 minutes; and 44.5 ± 9.2 nm•sec^{-1}, 10% CO_2 plus methazolamide period, n=6, Table 3-6), although V_T was significantly was significantly changed following exposure to 10% CO_2 and methazolamide (-35.0 ± 11.6 mV, 5% CO_2 period; -44.2 ± 14.0 mV, exposure to 10% CO_2 from 30 to 60 minutes; -46.9 ± 14.7 mV, exposure to 10% CO_2 from 60 to 90 minutes; and -50.7 ± 14.5 mV, 10% CO_2 plus methazolamide period, n=6, Table 3-6). Because Oberleithner and his associates (1990) have demonstrated that H-K-ATPase requires K conductance for its function, and Ba secondarily inhibits H-K-ATPase in Madin Darby Canine Kidney (MDCK) cell, the CCD was alternatively perfused in the absence of luminal Ba. As shown in Table 3-6, 10% CO_2 did not have trend to stimulate Rb efflux (73.1 ± 37.9 nm•sec^{-1}, 5% CO_2 period; 66.9 ± 40.1 nm•sec^{-1}, exposure to 10% CO_2 from 60 to 90 minutes; and 64.4 ± 32.2 nm•sec^{-1}, exposure to 10% CO_2 from 90 to 120 minutes, n=2, Table 3-6).

3.4 Discussion

3.4.1 Effect of 10% CO_2

To our knowledge, the present studies provide the direct evidence that respiratory acidosis (10% CO_2) stimulates K absorptive flux, assessed as ^{86}Rb lumen-to-bath efflux coefficient (K_{Rb}), in the CCD of K-restricted rabbits. Moreover, this enhanced K_{Rb} appears mediated by an H-K-ATPase. The rapid response to 10% CO_2 suggests that the stimulus increases either the H-K-ATPase units in the apical membrane or the kinetics of this pump. Exocytosis of H-K-ATPase to the apical membrane in response to the several stimuli have been demonstrated in the gastric gland (Forte et al., 1981). In addition, CO_2 has been shown to be lack of major effects on kinetics of H pumps in turtle

urinary bladder (Schwartz and Al-Awqati, 1986). The present studies are consistent with the hypothesis that 10% CO_2 stimulates the exocytotic insertion of H-K-ATPase to the apical membrane of the collecting duct. 10% CO_2 stimulates H secretion in gastric gland (Kidder and Montgomery, 1974). Wingo (1989) and other investigators (Planelles et al., 1991) have shown that renal H-K-ATPase is responsible not only for K absorption, but also for H secretion. Indeed, microcatheterization studies in rats with acute respiratory acidosis demonstrated that acidification was augmented prior to the inner medullary collecting duct (Bengele et al., 1984). From the in vitro microperfusion studies, Laski and Kurtzman (1990) have observed that total CO_2 flux was significantly enhanced by the CCD after the rabbits were exposed to elevated CO_2 tension chamber, indicating that the CCD increases its acidification rate in response to hypercapnia. More direct evidence supporting the positive role of CO_2 in acidification comes from McKinney and Davidson (1988) *in vitro* perfusing tubule study. They have found that total CO_2 absorption was profoundly increased by the CCD following exposure to 15% CO_2 for at least 20 minutes. On the other hand, by acute reduction of peritubular pCO_2 from 40 to less than 14 mmHg, Jacobson (1984) has shown that bicarbonate absorption significantly decreased by medullary collecting duct. However, some studies were not able to demonstrate stimulatory effect of CO_2 on acidification (Breyer et al., 1986; Lucci et al., 1982). We have no mechanism to reconcile these data. It is pertinent to ask whether the H-K-ATPase, or H-ATPase, or both, mediate this effect. The application of the specific inhibitor for each enzyme would shed more light on this question. Nevertheless, exocytotic insertion process has been proposed to be a strong candidate to mediate this enhancement in

acidification by increase in tension of CO_2 (McKinney and Davidson, 1988). The observations made at cellular level further strengthes such a hypothesis H-K-ATPase has been localized by immunocytochemistry to the intercalated cell in the CCD and outer medullary collecting duct (Wingo et al., 1989 & 1990). Madsen and Tisher (1983) have quantified that respiratory acidosis increases the surface density of the apical membrane concomitantly with decrease in the number of tubulovesicular profiles in the apical region of the intercalated cell of rats. Schwartz and Al-Awqati (1985), using fluorescent dextran assay, have directly demonstrated that CO_2 stimulates exocytotic fusion of the vesicles in the proximal straight tubule and collecting duct of rabbits. Turtle urinary bladder has many transport characteristics similar to collecting duct. Exposure of CO_2 also increases H secretion in this epithelia, although H-ATPase has been interpreted to be responsible in part for this stimulation (Cannon et al., 1985; Schwartz and Al-Awqati, 1986; Steinmetz, 1986; Stetson and Steinmetz, 1983 & 1986; Van Adelsberg and Al-Awqati, 1986). However, recent studies have also demonstrated the presence of K-ATPase activity in this epithelia (Husted and Steinmetz, 1981; Sharma et al., 1991). Exocytosis of the pumps has been repeatedly shown to mediate this response (Cannon et al., 1985; Schwartz and Al-Awqati, 1986; Steinmetz, 1986; Stetson and Steinmetz, 1983; Van Adelsberg and Al-Awqati, 1986).

The cell swelling has been observed following exposure to 10% CO_2 (Figure 3-12), whereas the change in morphology of the tubules was not able to be observed if the stimulatory effect of 10% CO_2 on Rb efflux was inhibited either by colchicine (Figure 3-13) or by MAPTAM (Figure 3-14). These observations suggest that the renal H-K-ATPase participates in cell volume

regulation. Two possible mechanisms may explain the effect of H-K-ATPase on cell volume. 10% CO_2 stimulates H-K-ATPase thereby increasing K/Rb entry from the apical membrane. It has been well known that acidosis inhibits K conductance or K channels (Boundry et al., 1976; Wang et al., 1990; Wright and Giebisch, 1992). It is possible that 10% CO_2 inhibits the K exit at the basolateral membrane. The intracellular K level increases from the these two additive effects. As a result, the osmolarity gradient is established. Alternatively, the renal H-K-ATPase can transport Na as demonstrated in Chapter 5. 10% CO_2 stimulates H-K-ATPase presumably increasing Na entry from the apical membrane. Acidosis inhibits Na-K-ATPase thereby inhibiting Na exit at the basolateral membrane (Wright and Giebisch, 1992). As a result, the osmolarity gradient is established.

As shown in Figure 3-1, each individual tubule responded to 10% CO_2 differently. Some tubule had big response in terms of Rb efflux stimulation upon exposure to 10% CO_2, whereas others did not. Rb efflux response to the stimulus is not correlated with voltage response. Whether the variation in K_{Rb} response to 10% CO_2 is due to the different populations of the CCD or the biological variation among the tubules is not clear. In fact, 20% CCD comes from the juxtamedullary nephron, whereas 80% CCD comes from the superficial nephron. Whether these two different populations of the CCD have different physiological functions in terms of potassium transport remain unknown.

3.4.2 Effect of Methazolamide

Exposure to CO_2 has been demonstrated to decrease intracellular pH, and this results in increase intracellular Ca level in turtle urinary bladder, although the effect of CO_2 on intracellular Ca activity varies in different cell

types (Cannon et al., 1985). Exocytotic fusion of H pumps is pH- and Ca-dependent in turtle urinary bladder. In this tissue decrease in tracellular pH results in an increase in intracellular Ca activity by CO_2 stimulates this insertion process (Cannon et al., 1985; Van Adelsberg and Al-Awqati, 1986). Acetazolamide, another inhibitor of carbonic anhydrase, has been shown to alkaline intracellular pH and decrease intracellular Ca activity (Van Adelsberg and Al-Awqati, 1986). The increase in intracellular pH and decrease in intracellular Ca level prevents the exocytotic insertion of acidic cytoplasmic vesicles containing H pumps into the apical membrane of turtle urinary bladder (Cannon et al., 1985; Schwartz and Al-Awqati, 1986; Steinmetz, 1986; Stetson and Steinmetz, 1983; Van Adelsberg and Al-Awqati, 1986). Consistent with the insertion hypothesis for H-K-ATPase, simultaneous exposure to 10% CO_2 and methazolamide prevented the stimulatory effect of 10% CO_2 on Rb efflux presumably because the alkalinization of intracellular pH by methazolamide inhibited the insertion cascade, whereas after 10% CO_2 enhanced Rb efflux presumably activated the exocytotic process of H-K-ATPase, subsequent addition of methazolamide had no detectable inhibition on Rb efflux (Fig. 3-4). In addition, methazolamide did not significantly affect Rb efflux when the CCD was perfused in the presence of 5% CO_2 throughout the experiments. This suggests that methazolamide-insensitive H source could be as a substrate for H-K-ATPase. Recently, the observation made by Sharma et al. (1991) suggests that not all H-K-ATPase depends on carbonic anhydrase-originated H in turtle urinary bladder. They have demonstrated that 1 mM acetazolamide applied to the serosal solution only reduced proton secretion to 30% of control, the subsequent addition of SCH28080 caused H secretion fall to zero. When

SCH28080 was given first, proton secretion decreased to 70% of control, and subsequent addition of acetazolamide reduced proton secretion to zero. Carbonic anhydrase-dependent acidification has been repeatedly demonstrated in the proximal tubule (Chan et al., 1983; Cogan et al., 1979; DuBose and Lucci, 1983). In contrast, whether all of acidification in the collecting duct is carbonic anhydrase-dependent remains controversial. Several groups have demonstrated that the collecting duct is able to maintain almost completely normal acidification rate after administration of acetazolamide both *in vivo* and *in vitro*, suggesting that a substantial carbonic anhydrase-independent H source is present in the collecting duct (Cogan et al., 1979; DuBose and Lucci, 1983; Frommer et al., 1984; Laski, 1987; McKinney and Davidson, 1987). However, the inhibitory effect of acetazolamide on acidification in the collecting duct has been reported by other investigators (Lombard et al., 1983). It is difficult to reconcile these observations at present time. Carbonic anhydrase-independent acidification may not be an unique phenomenon only observed in collecting duct. As early as in 1958, Durbin and Heinz (1958) have shown that carbonic anhydrase is not essential for H secretion in gastric gland. Acidification by turtle urinary bladder has been also reported to be in part independent of carbonic anhydrase (Schilb and Brodsky, 1966; Schwartz et al., 1972). However, the sources of these protons remain speculative. At least two attractive mechanisms have has been proposed to explain the carbonic anhydrase-independent H sources which could be from ATP hydrolysis as suggested for gastric H-K-ATPase (Ljungstrom et al., 1984), or non-catalyzed hydration of CO_2 (Maren, 1974).

Methazolamide has been repeatedly demonstrated to make V_T more lumen-negative in the present studies which is consistant with other reports(Koeppen, 1989; Koeppen and Helman, 1982; Lombard et al., 1983; McKinney and Davidson, 1987). However, the mechanisms underlying this effect is not clear. This effect could be due to inhibition of electrogenic H secretion , or influence of anion transport (Koeppen, 1989; Koeppen and Helman, 1982), or both.

3.4.3 Effects of Colchicine, MAPTAM, and W-7

Much of our knowledge about the role of microtubules, intracellular Ca, and calmodulin in the stimulation of H secretion (presumebly in part via H-K-ATPase) is derived from the observations made in the turtle urinary bladder and collecting duct. Pretreatment with colchicine inhibits exocytotic fusion of proton pumps to the apical membrane and the increase in the apical surface density and proton secretion by the increase in CO_2 tension in the turtle urinary bladder, whereas lumicolchicine, which is structually similar to colchicine but does not bind to tubulin, had no effect (Stetson and Steinmetz, 1983). More directly, Schwartz and Al-Aqwati (1985) demonstrated that colchicine prevented the insertion process of proton pumps on exposure to CO_2 in the proximal tubule and cotical and outer medullary collecting duct. McKinney and Davidson (1988) later reported that colchicine inhibited total CO_2 absorption in response to 15% CO_2 at least by the inner strip of the outer medullary collecting duct, whereas lumicolchicine had no effect. Recently, Brown et al. (1991) have shown that colchicine scatters the proton pumps in the cytoplasma of the renal epithelial cells. The soluble GTP-binding proteins have been suggested to be in control of the direction of insertion (Bourne, 1988; Hall, 1990). If H-K-ATPase

is involved in the stimulation of H secretion upon exposure to CO_2, colchicine may also inhibit K absorption. Based on these observations and hypothesis, we selectively minupulated the conditions which were expected to inhibit microtubule functions at two different stages. When the CCD was simultaneous exposed to 10% CO_2 and colchicine, colchicine inhibited the stimulation of Rb efflux by 10% CO_2. In contrast, after Rb efflux increased by 10% CO_2, subsequent addition of colchicine had no significant effect. These results are consistant with the dependence of the increased Rb efflux on exocytosis of H-K-ATPase, which is mediated by the rearrangement of microtubule. The voltage became more lumen-negative during 10% CO_2 and 10% CO_2 plus colchicine periods, which is in contrast to the initial studies. We have no precise explaination for this observation but speculations. The baseline of voltage of these two sets of experiments was more lumen-negative than that of the previous studies. This sugggests that the tubules used in these two sets of experiments characterize more similarly to normal tubules in which voltage became more lumen-negative upon exposure to 10% CO_2.

Intracellular calcium is respobsible for a variety of cellular events to numerous stimuli such as activation of gastric H-K-ATPase elicited by carbachol and gastrin and of H-ATPase in turtle urinary bladder induced by carbon dioxide (Forte et al., 1981). It has been believed that many Ca responses result from protein phosphorylation or dephosphorylation through the reactions with calmodulin. For instance, calmodulin regulating Ca-dependent microtubule assembly and disassembly has been illustrated in several types of cells (Marcum et al., 1978). A great deal of demonstrations have been evidently suggestive that exocytotic fusion of acidic vesicles containing H-ATPase is Ca-dependent process

in turtle urinary bladder (Cannon et al., 1985; Schwartz and Al-Awqati, 1986; Van Adelsberg and Al-Awqati, 1986). Exposure to CO_2 increases intracellular Ca activity in tissue (Cannon et al., 1985). This elicits whole cascade of insertion process. When the increase in intracellular Ca activity is minimized, either by chelation of extracellular Ca (Van Adelsberg and Al-Awqati, 1986), or by buffering intracellular Ca (Cannon et al., 1985; McKinney and Davidson, 1988), the excytosis and the increased proton secretion are mitigated both in the turtle urinary bladder and the collecting duct. A body of evidence is also consistant with a mediator role for calmodulin in the stimulation of H secretion resulting from exposure to CO_2. The increment in total CO_2 absorption upon exposure to CO_2 by the inner strip of outer medullary collecting duct is markedly attenuated by the potent antaganizer of calmodulin (W-7) but not by a structurally related analogue with less calmodulin inhibitory effect (W-5) (Dytko and Arruda, 1985; McKinney and Davidson, 1988). In view of these observations, we have chosen two maneuvers expected to be associated with alterations in intracellular Ca or calmodulin activity. Pretreatment with 0.5μM MAPTAM blocked the stimulatory effect of Rb efflux by 10% CO_2. The result is presumably due to the failure of intracellular ionized Ca level to rise. Moreover, pretreatment with 0.5μM W-7 totally abolished the increase in Rb efflux by 10% CO_2. However, the significant decrease in Rb efflux was observed during W-7 period. The mechanisms explaining this phenomenon remain to be elucidated. W-7 may have additional inhibition of Rb efflux mediated by K conductance. The important point here is that W-7 prevented the increase in Rb efflux induced by 10% CO_2, providing the clear evidence indicating the role of calmodulin in this response. MAPTAM significantly made voltage more

lumen negative, whereas W-7 only had a trend. Many factors contribute and influence voltage. The mechanisms of these observations remain speculutive. It is possible that MAPTAM and W-7 inhibit electrogenic H-ATPase, which has been demonstrated in the turtle urinary bladder (Dytko and Arruda, 1985; Schwartz and Al-Awqati, 1985 & 1986; Van Adelsberg and Al-Awqati, 1986).

3.4.4 Effects of Basolateral Ba on K-restricted CCD and 10% CO_2 on Normal CCD

A large Ba-sensitive K conductance has been identified in the basolateral membrane of both principal cell and intercalated cell of rat (Schlatter and Schafer, 1987). However, electrophysiological studies conducted by Muto et al. (1987) suggest that little K conductance is present in the basolateral membrane of intercalated cell of K-replete rabbit CCD. Our present studies indicate that a Ba-sensitive pathway is present in the basolateral membrane of K-restricted CCD, and this pathway participates in K/Rb absorptive process. Alternatively, it may reflect an effect of Ba on non-conductive K exit as proposed by Greger and Schalatter (1983) for the thick ascending limb of Henle. It is reasonable to posit that under K-restriction, this pathway exerts its function to meet the need for K conservation, whereas this pathway may not function under K replete conditions. Consistantly with such a hypothesis, 10% CO_2 had no significant effect on Rb efflux by normal CCD regardless of the presence or absence of luminal Ba. Under K-replete condition, the main function of H-K-ATPase may be secret proton, whereas the absorbed K by this enzyme leaks back to the lumen because of the functionally closed basolateral K exit pathway. Rats conserve K more efficiently than rabbits. Whether the species-difference in conservation of K is due to the basolateral K exit is unknown at present time. The alternative possibility is that the stimulatory effect of 10% CO_2 on

Rb efflux through H-K-ATPase is overshadowed by the inhibitory effect on Rb efflux via K-conductive pathway when the tubules were perfused in the absence of luminal Ba. Ba may secodary inhibit H-K-ATPase when the CCD was perfused in the presence of luminal Ba. The precedent suggestion is derived from Oberleithner et al. (1990) observation made in MDCK cell. They have shown that Ba and omeprazole inhibit short-circuit current and acidification of the surface of the apical side of the dome. The effect of Ba is attributable to inhibition of K recycle at the apical membrane thereby inhibits H-K-ATPase in this type of cells.

3.5 Summary

In summary, these studies demonstrate that 10% CO_2 profoundly stimulates Rb efflux, and this stimulation was totally abolished by SCH28080, suggesting that an H-K-ATPase mediates this process in the K-restricted CCD. The rapid response to 10% CO_2 implies that this maneuver increases the luminal activity of existing H-K-ATPase pump units. The parallel changes in V_T regardless of the absence or presence of SCH28080 reflect that the change in V_T does not account for the effect of 10% CO_2 on Rb efflux. The subsequent addition of methazolamide after 10% CO_2 had no significant effect on Rb efflux, whereas simultaneous exposure of the tubules to 10% CO_2 and methazolamide prevented the enhancement of Rb efflux by 10% CO_2, suggesting that carbonic anhydrase is not necessary for maintaining activation of H-K-ATPase by 10% CO_2, but is necessary for initiating activation of H-K-ATPase. Colchicine, MAPTAM, and W-7 inhibit the stimulation of Rb efflux by 10%CO_2, implying that activation of H-K-ATPase is dependent on the functional microtubules, increase in intracellular Ca activity, and functional calmodulin and

posssiblly mediated by exocytotic process. Basolateral Ba totally blocked the enhancement of Rb efflux by 10% CO_2, indicating that a Ba-sensitive exit pathway mediates the stimulation of K_{Rb} by 10% CO_2.

Table 3-1. Composition of solutions (in mM)

	Solution A	Solution B	Solution C
Na	135	135	135
K	5	5	5
Cl	110.4	114.4	114.4
Ca	1.2	1.2	1.2
Mg	1	1	1
Acetate	10	10	10
Glucose	8	8	8
Alanine	5	5	5
HEPES	10	10	10
Gluconate	23	23	23
Ba	0	2	4
TMA	4	4	0
Osmol (m Osm)	317.6	322.6	321.6

Table 3-2. Effect of luminal 0.1% DMSO on K_{Rb} and V_T

	Basal	DMSO
K_{Rb} ($nm \cdot sec^{-1}$)	81.4 ± 9.0	83.0 ± 13.7
V_T (mV)	-8.3 ± 3.4	-5.8 ± 4.1

DMSO, dimethylsufoxide (n=6).

Table 3-3. Time control for K_{Rb} and V_T

Time after decapitation (in minutes)	100-150	150-200	200-250	250-300
K_{Rb} ($nm \cdot sec^{-1}$)	82.1 ± 13.0	83.6 ± 11.7	106 ± 8.8	100 ± 15.1
V_T (mV)	4.2 ± 3.4	3.9 ± 3.1	5.5 ± 2.6	5.4 ± 2.3

(n=5).

Table 3-4. Effect of 10% CO_2 on V_T in the presence of colchicine.

	5% CO_2	10% CO_2 + Colchicine
V_T (mV) (n=7)	-12.9 ± 4.9	-15.6 ± 5.0

	5% CO_2	10% CO_2	10% CO_2 + Colchicine
V_T (mV) (n=7)	-11.2 ± 7.1	-16.9 ± 9.4[a]	-17.4 ± 9.5[b]

[a] $p < 0.05$, vs 5% CO_2 period;
[b] $p < 0.01$, vs 5% CO_2 period.

Table 3-5. Effect of 10% CO_2 on V_T in the tubules pretreated with 0.5 μM MAPTAM and W-7.

	MAPTAM	
	5% CO_2	10% CO_2
V_T (mV) (n=6)	-15.7 ± 5.9	-23.9 ± 6.8*
	W-7	
	5% CO_2	10% CO_2
V_T (mV) (n=6)	-12.2 ± 3.9	-14.4 ± 3.5

* $p<0.05$, vs 5% CO_2 period.

Table 3-6. Effect of 10 CO_2 on the normal CCD in the presence and absence of luminal Ba

	With Ba	(n=6)		
	5% CO_2	10% CO_2(30-60 minutes)	10% CO_2(60-90 minutes)	10%CO_2+Methazolazmide
K_{Rb} ($nm \cdot sec^{-1}$)	48.1 ± 7.2	42.5 ± 8.3	44.6 ± 7.5	44.5 ± 9.2
V_T (mV)	-35.0 ± 11.6	-44.2 ± 14.0[a]	-46.9 ± 14.7[a]	-50.7 ± 14.5
	Without Ba	(n=2)		
	5% CO_2	10% CO_2(60-90 minutes)	10% CO_2(90-120 minutes)	
K_{Rb} ($nm \cdot sec^{-1}$)	73.1 ± 37.9	66.9 ± 40.1	64.4 ± 32.2	
V_T (mV)	-33.4 ± 0.4	-32.5 ± 0.2	-20.4 ± 10.4	

[a] $p<0.05$, vs the 5% CO_2 period.

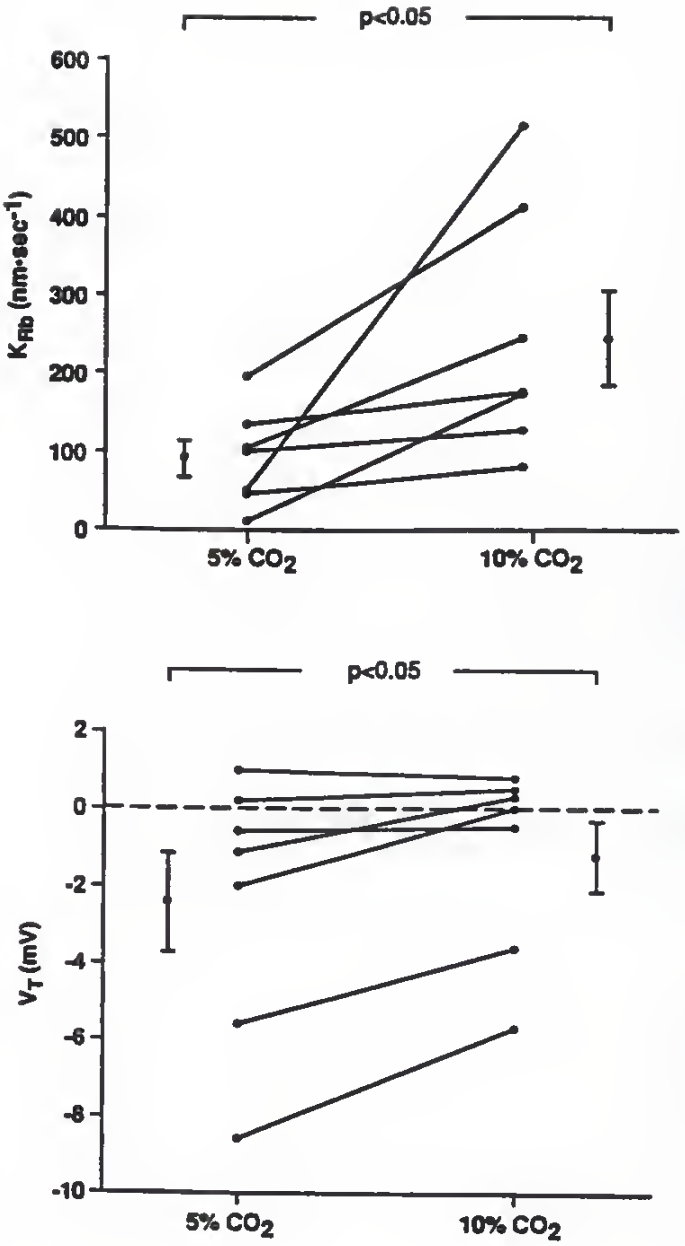


Figure 3-1. Effect of 10% CO_2 on K_{Rb} (top) and V_T (bottom) in the absence of SCH28080 (n = 7). The tubules were allowed equilibrating for one hour before starting collection of sample during 10% CO_2 period. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

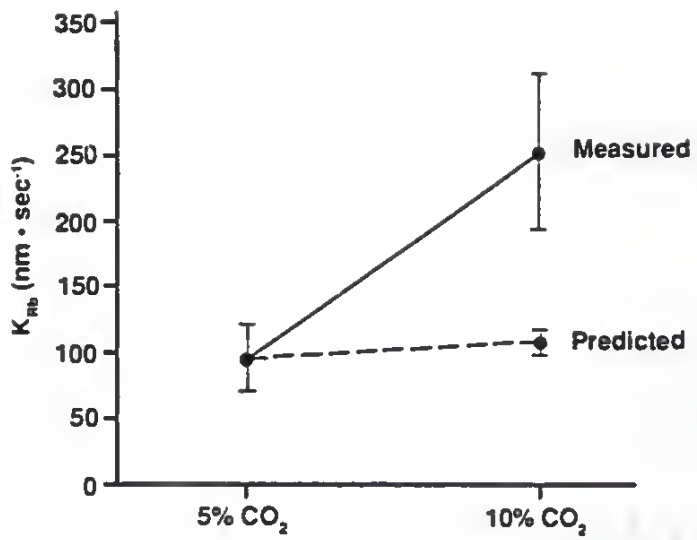


Figure 3-2. The predicted voltage-mediated increase in K_{Rb} and observed increase in K_{Rb} following exposure to 10% CO_2. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

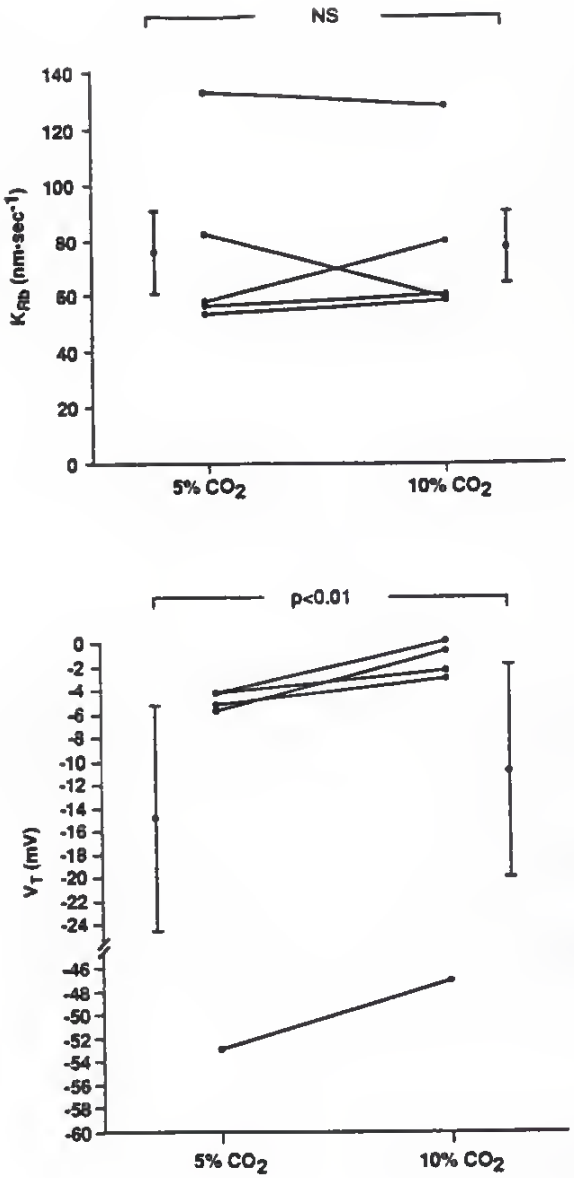


Figure 3-3. Effect of 10% CO_2 on K_{Rb} (top) and V_T (bottom) in the presence of SCH28080 (n =5). The tubules were allowed equilibrating for one hour before starting collection of sample during 10% CO_2 period. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient V_T, transepithelial voltage.

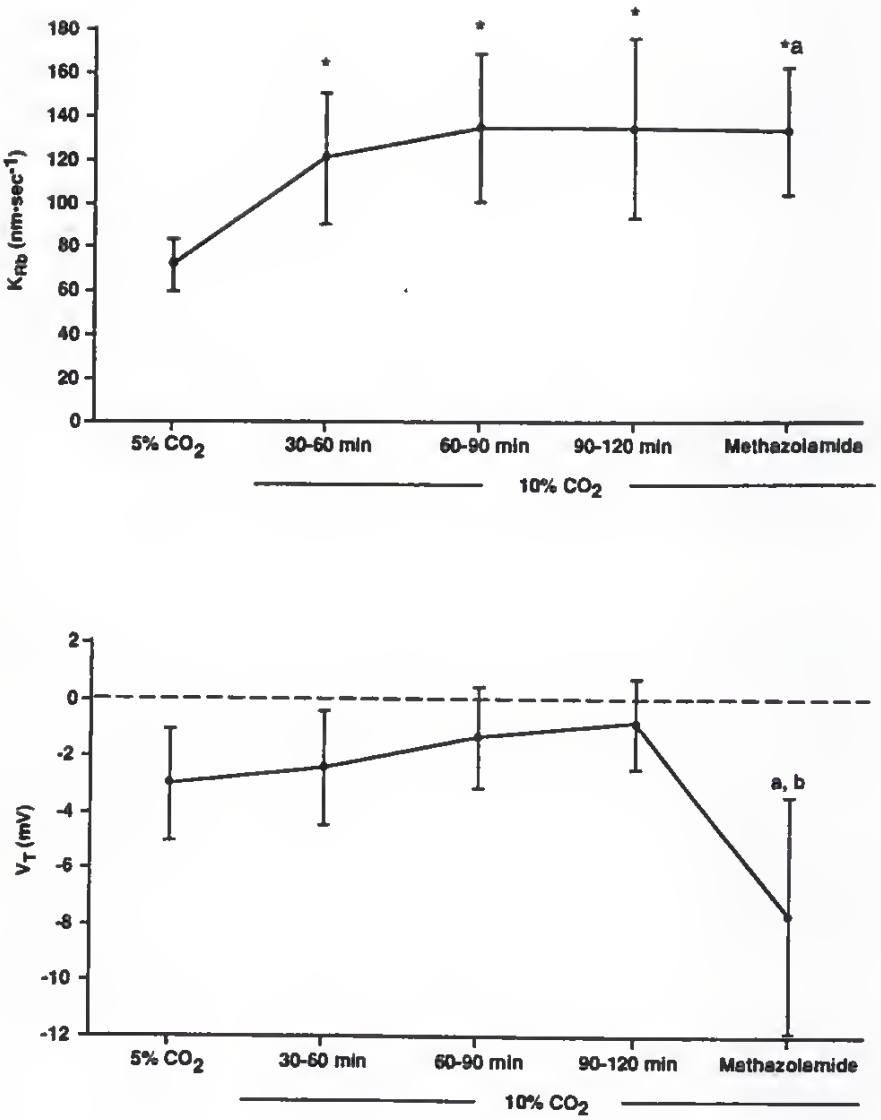


Figure 3-4. Effect of 10% CO_2 and 0.1 mM methazolamide on K_{Rb} and V_T in the absence of SCH28080 (n = 6). [a]$P < 0.05$ compared with 5% CO_2 period. [a]$P = 0.06$ compared with 60-90 minute period. [b]$P < 0.05$ compared with 90-120 minute period. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

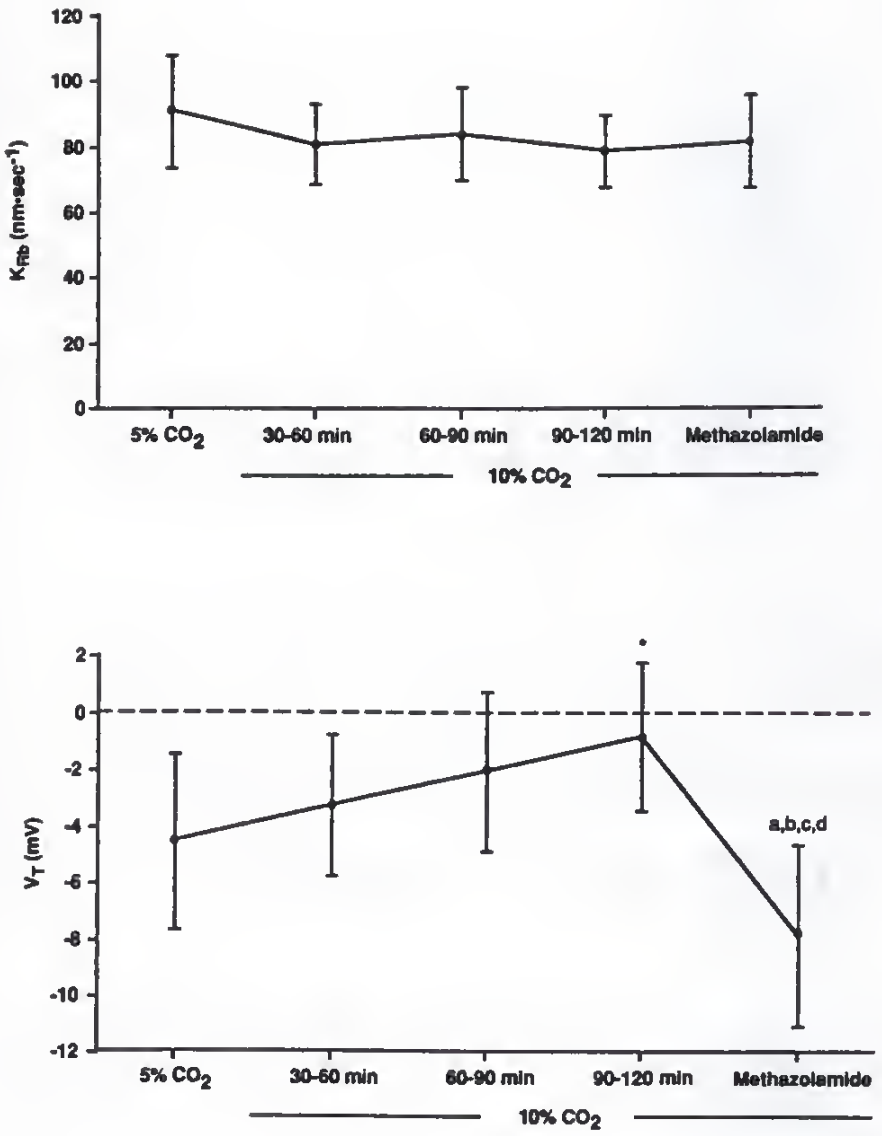


Figure 3-5. Effect of 10% CO_2 and 0.1 mM methazolamide on K_{Rb} (top) V_T (bottom) in the presence of SCH28080 (n = 7). $^{a}P < 0.05$ compared with 5% CO_2 period. $P < 0.01$ compared with 30-60 minute period. $^{b}P < 0.001$ compared with 90-120 minute period. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

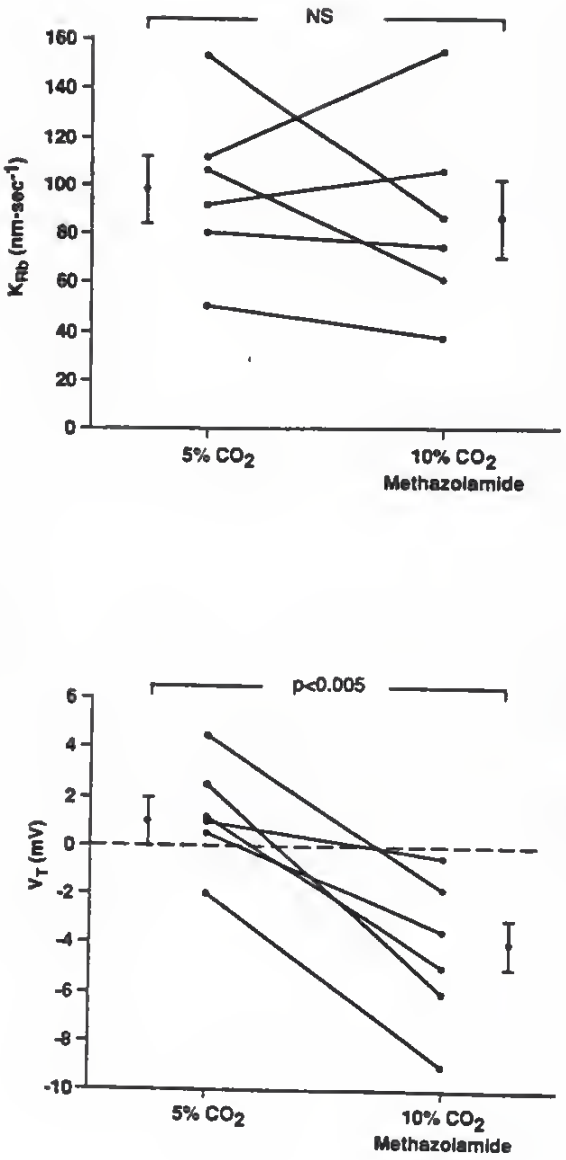


Figure 3-6. Effect of simultaneous exposure to 10% CO_2 and 0.1 mM methazolamide on K_{Rb} (top) and V_T (bottom) (n = 6). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

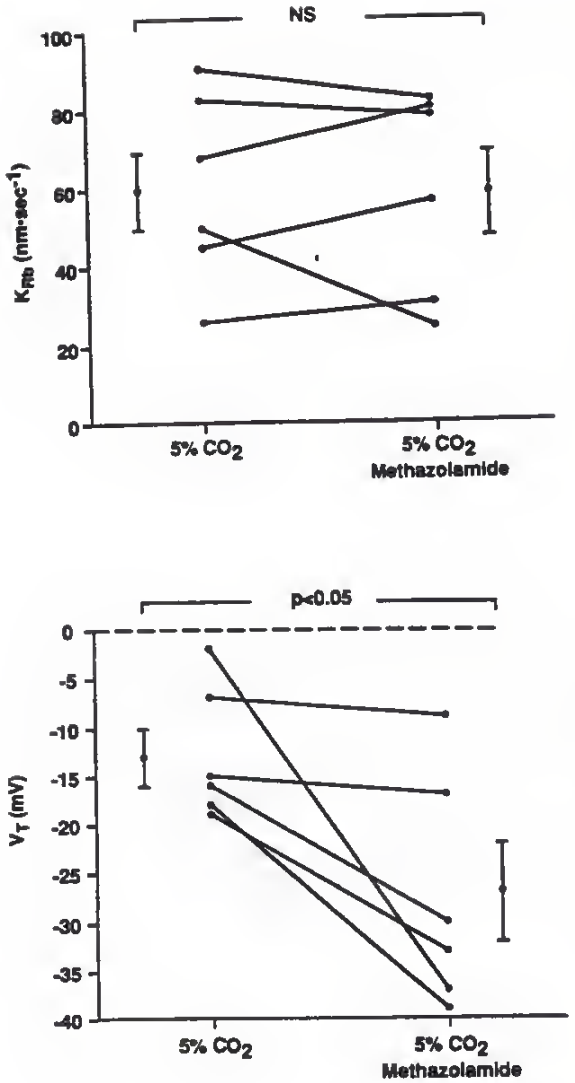


Figure 3-7. Effect of 0.1mM methazolamide on K_{Rb} (top) and V_T (bottom) in the presence of 5% CO_2 (n = 6). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

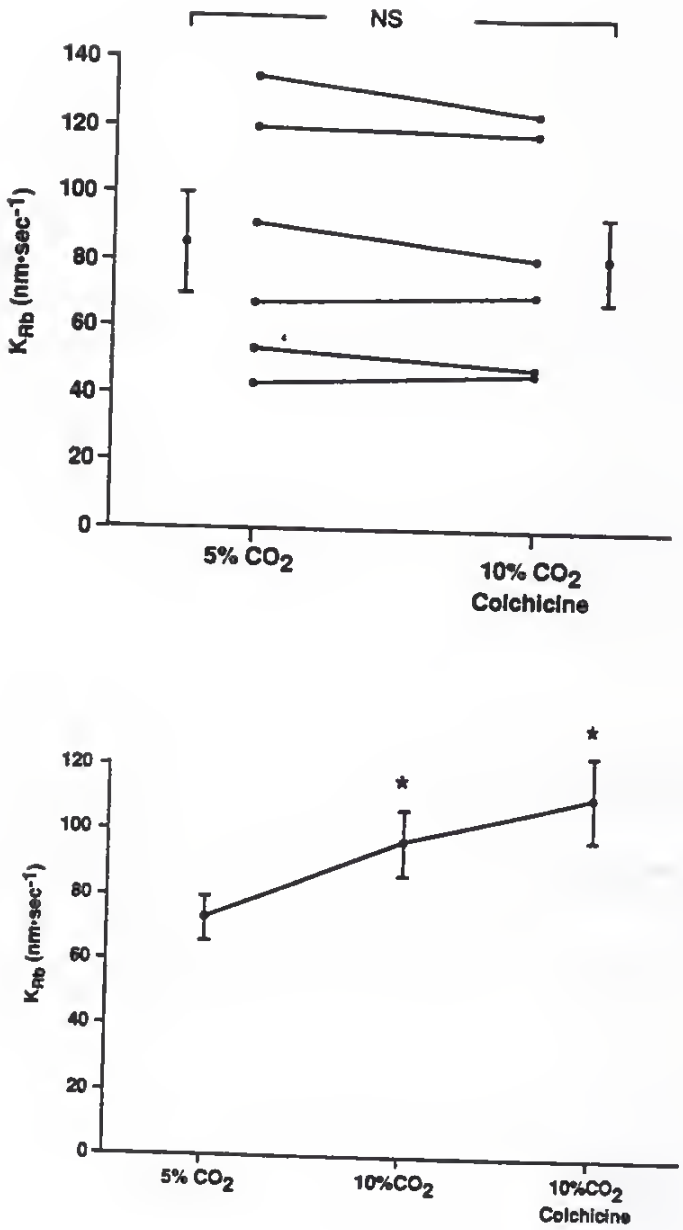


Figure 3-8. Effect of simultaneous exposure to 10% CO_2 and 0.5 mM colchicine (top) and exposure to colchicine after 10%CO_2 (bottom) on K_{Rb} (n = 6). ^{86}Rb lumen-to-bath efflux coefficient.

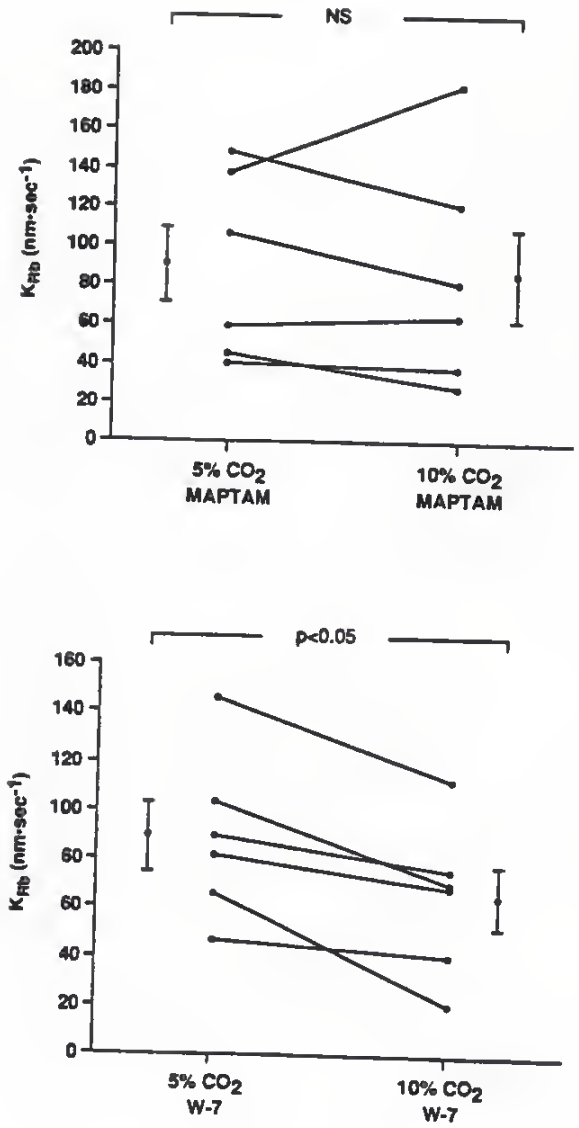


Figure 3-9. Effect of 10% CO_2 on K_{Rb} by the tubules pretreated with 0.5μM MAPTAM (top) and W-7 (bottom)(n = 6). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

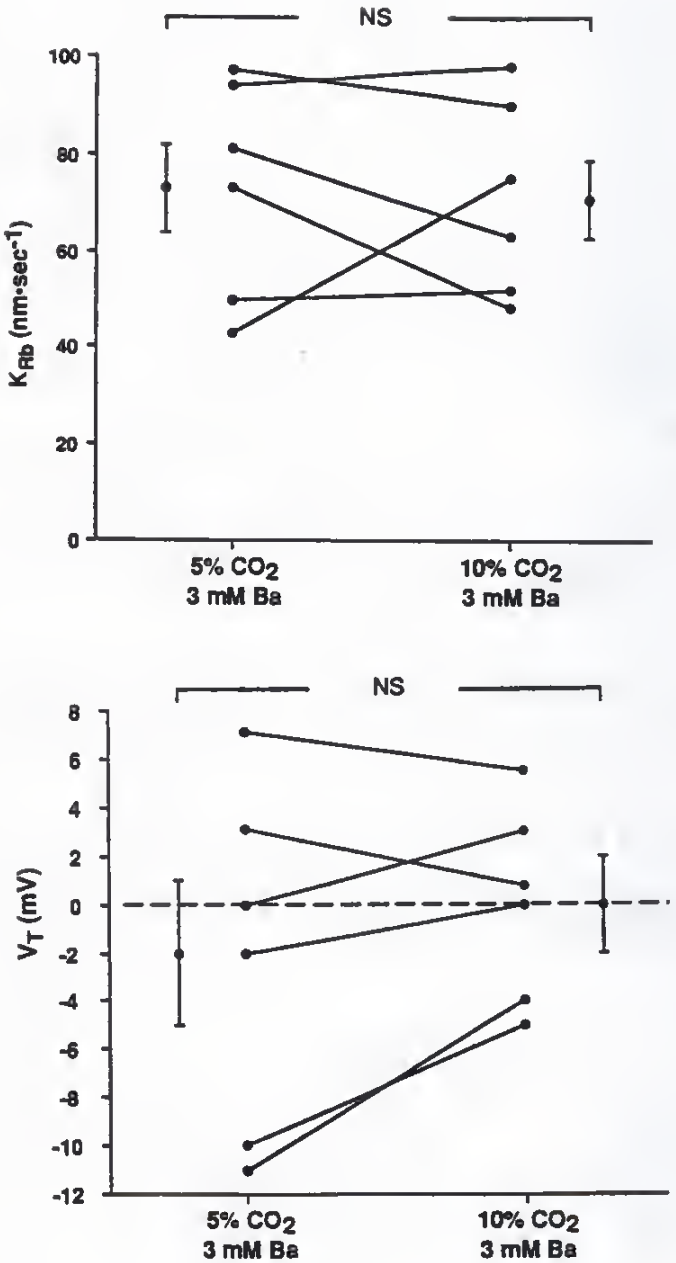


Figure 3-10. Effect of peritubular 3 mM Ba on K_{Rb} (top) and V_T (bottom) (n = 6). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

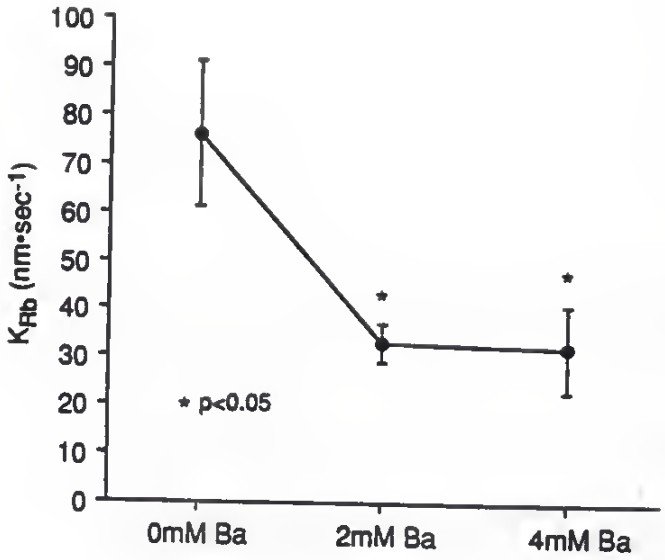


Figure 3-11. The dose-response curve of Ba on K_{Rb}. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

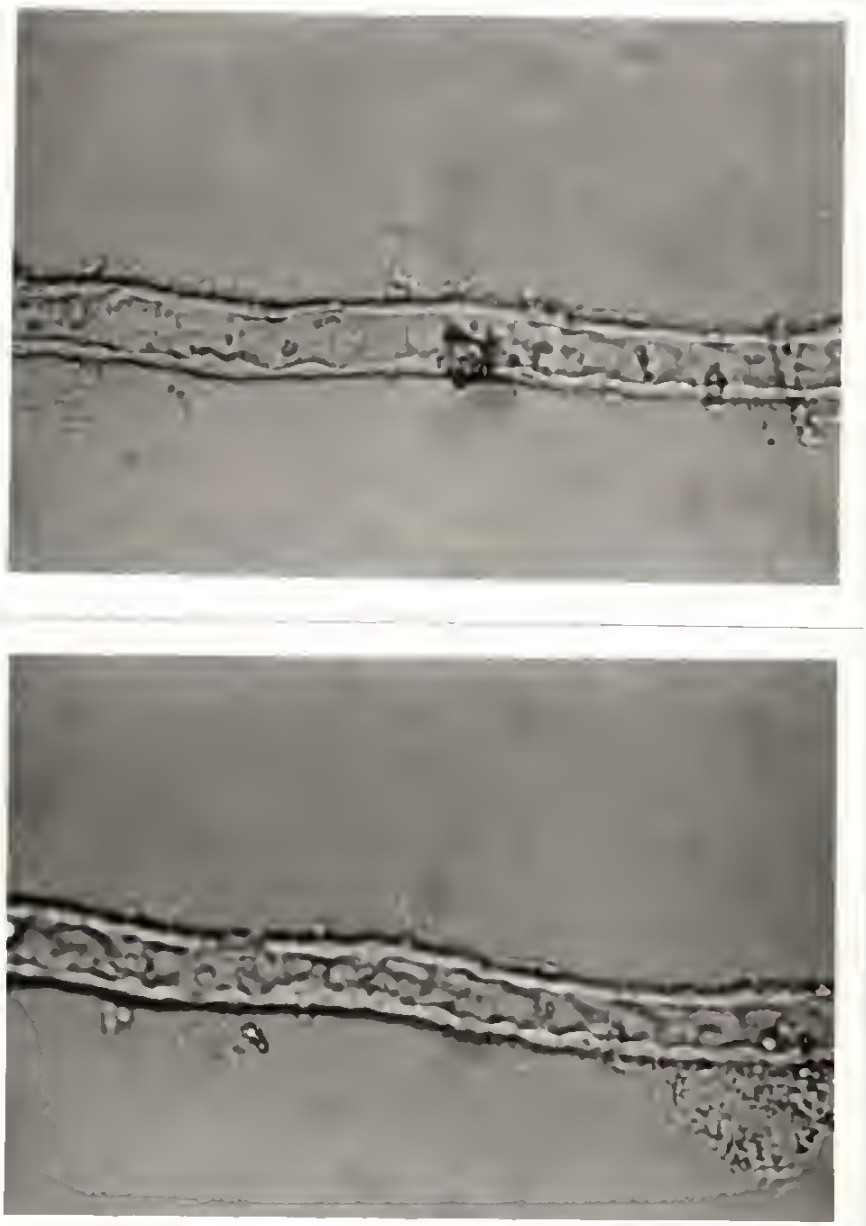


Figure 3-12. The CCD was swollen following expusure to 10% CO_2. Top: 5% CO_2 period, Bottom: 10% CO_2.

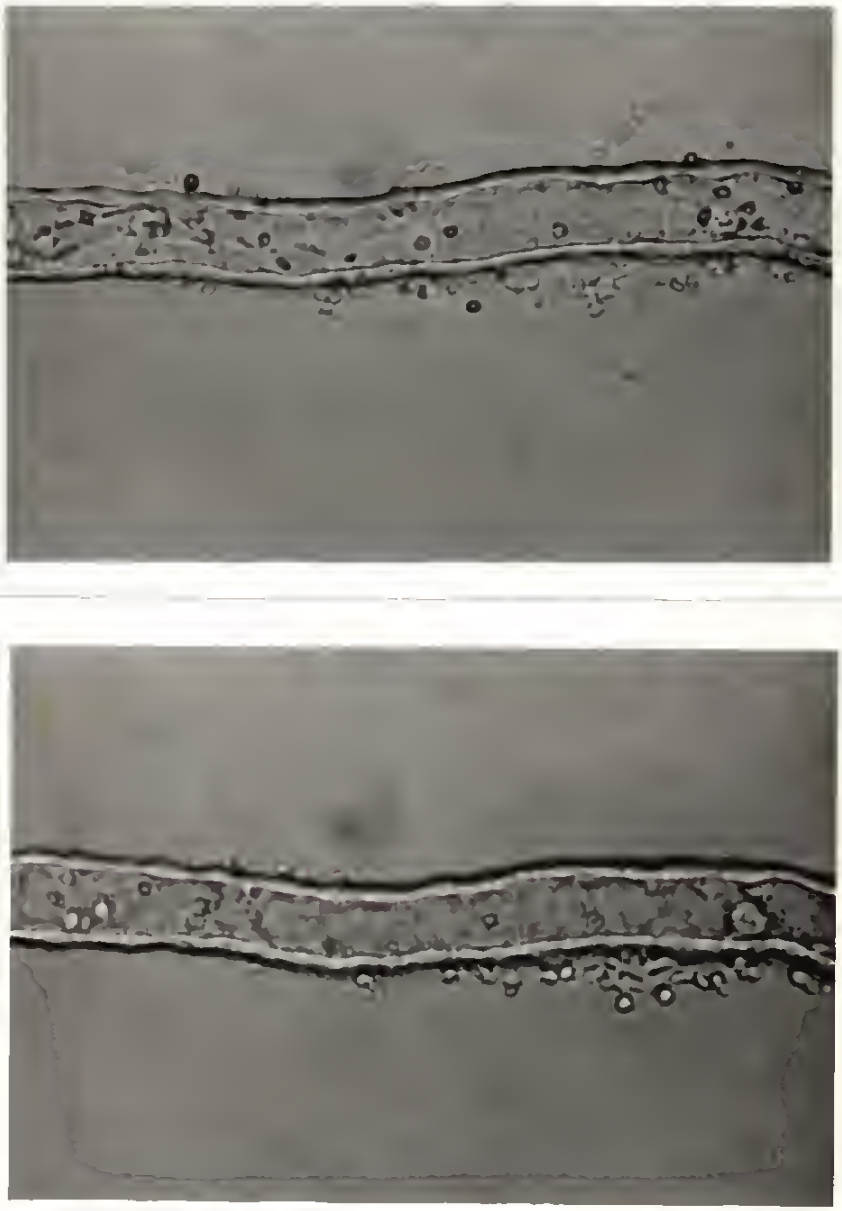


Figure 3-13. The simultaneous presence of 10% CO_2 and colchicine prevented the cell swelling. Top: 5% CO_2 period, Bottom: 10% CO_2 plus colchicine period.

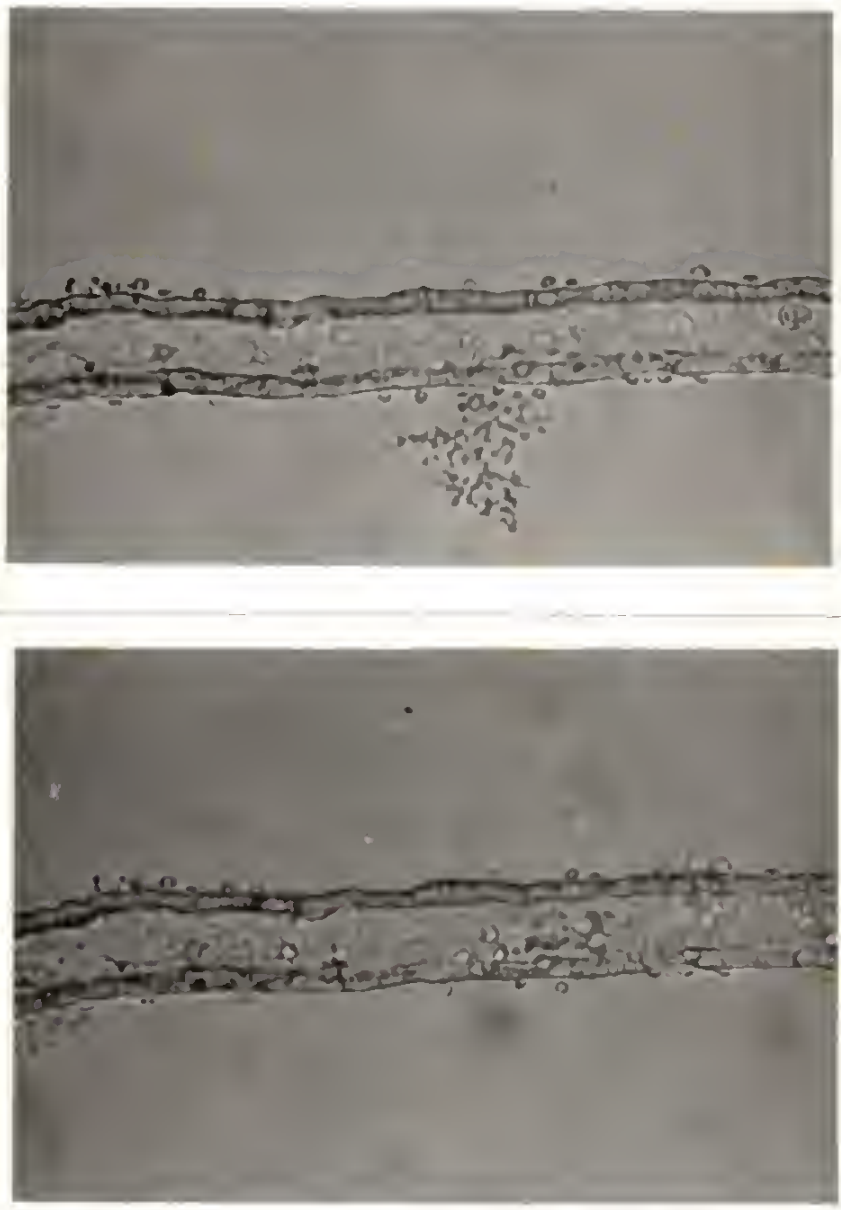


Figure 3-14. Effect of 10% CO_2 on cell swelling in the presence of MAPTAM. Top: 5% CO_2 period, Bottom: 10% CO_2.

CHAPTER 4
EFFECT OF BARIUM, AMILORIDE AND OUABAIN ON Rb EFFELUX

4.1 Introduction

There is general agreement that potassium (K) secretion by the cortical collecting duct (CCD) involves active translocation of K across the basolateral membrane via Na-K-ATPase and passive exit across the apical membrane, in part via a K-conductive pathway (Muto et al., 1988; Stokes, 1981; Warden et al., 1989; Wingo, 1984). Administration of luminal barium, an inhibitor of K-conductance, or amiloride, an inhibitor of conductive Na entry, or peritubular ouabain, an inhibitor of Na-K-ATPase, inhibits net K secretion (O'Neil and Boulpaep, 1979; Stoner et al., 1974; Wingo, 1984 & 1985). These facts suggest that K secretion is largely dependent on K exit through an Ba-sensitive pathway and Na entry through an amiloride-sensitive pathway at the apical membrane, and K uptake through Na-K-ATPase at the basolateral membrane.

Additionally, luminal amiloride has been demonstrated to substantially stimulate lumen-to-bath tracer K efflux, or tracer Rb efflux, used as a marker of K efflux in the *in vitro* microperfused CCD of both normal rabbits and DOCA-treated rabbits (Stokes, 1981; Warden et al., 1989). This effect depends on a Ba-sensitive K conductance. Therefore, the first objective of this studies is to examine whether Ba inhibits tracer Rb efflux, and whether the effect of amiloride on Rb efflux is preserved in K-restricted CCD.

Ouabain profoundly increases K absorption in gastric gland, and this stimulation has been attributed to H-K-ATPase (Reenstra et al., 1986). H-K-ATPase has been also identified in colonic epithelium which has many transport characteristics similar to the collecting duct (Kaunitz and Sachs, 1986). Serosal ouabain has been shown to not only inhibit K secretion, but also stimulate K absorption by this tissue (Halm and Frizzell, 1986; Turnamian and Binder, 1989). Therefore, the second objective of this studies is to address the issue whether peritubular ouabain increases Rb efflux, and whether this increment is mediated by an H-K-ATPase in the K-restricted CCD.

However, the issue of whether K absorption by the CD proceeds via an H-K-ATPase is not completely clear. During K restriction, Na-K-ATPase activity increases in the rat CD, although this increase in Na-K-ATPase activity is not accompanied by an increased [3H]-ouabain binding in intact tubules. However, in permeabilized tubules, [^{3}H]-ouabain binding does increase proportionately to the increase in Na-K-ATPase activity. These observations led Hayashi and Katz (1987) to hypothesize that Na-K-ATPase was sequestered in an inaccessible site, either an intracellular compartment or the luminal membrane. If Na-K-ATPase is present at the luminal membrane, it could participate in K absorption. In addition, mucosal ouabain has been shown to inhibit active K absorption in the rat and guinea pig colon (Perron and McBride, 1988; Sweiry and Binder, 1990; Suzuki and Kaneko, 1987 & 1989) and in the turtle bladder (Husted and Steinmetz, 1981). Thus, the third objective of the present studies was to determine whether a functional H-K-ATPase or a functional Na-K-ATPase was present at the apical membrane of the CCD of K-restricted rabbits.

4.2 Methods and Material

The tubules were dissected with Solution A (Table 4-1) containing additional 5% vol/vol feral calf serum. The bath solution which is identical to dissection solution was used throughout all sets of experiments. Three different perfusates (Solution A, Solution B or Solution C) were used as appropriate. All solutions were gassed to pH 7.4 with 95% O_2 and 5% CO_2. Amiloride was a gift of Merck Sharp & Dohme (Rahway, NJ) and was dissolved in the perfusate directly. SCH28080 (gift of Dr. James Kaninsky, Schering Corporation, Bloomfield, NJ) was dissolved in dimethyl sulfoxide (DMSO). Ouabain was dissolved in 0.9% NaCl solution.

4.3 Results

4.3.1 Protocol 1

To examine whether amiloride enhances K_{Rb} in the K-restricted animals, the first set of experiments was performed in the absence of luminal Ba. Thus, eight tubules were perfused with solution A, or solution A plus 1 mM amiloride designated as basal and amiloride periods, respectively. The order of the periods was rotated and there was no evidence of time-dependent effect on K_{Kb} or V_T. In the absence of luminal Ba, luminal 1 mM amiloride significantly increased K_{Rb} from 90.7 ± 14.3 $nm \cdot sec^{-1}$ (basal period) to 119 ± 20.6 $nm \cdot sec^{-1}$ (amiloride period, $P < 0.05$, Figure 4-1), and V_T from 0.6 ± 1.5 mV (basal period) to 4.7 ± 0.9 mV (amiloride period, $P < 0.05$, Figure 4-2). To examine whether the effect of amiloride on Rb efflux was mediated by Ba-sensitive pathway, the second set of experiments was performed in the presence of luminal 2 mM Ba, because in view of the voltage-dependent nature of Ba inhibition, Rb efflux in the K-restricted CCD should be maximally inhibited by 2 mM Ba at which has been demonstrated to maximally inhibit

K_{Rb} by normal CCD. To determine whether tracer Rb efflux via the H-K-ATPase was demonstrable in the presence of a maximum pharmacologic concentration of amiloride, 10 μM SCH28080 was applied to the perfusate containing 1 mM amiloride and 2 mM Ba during the third period. Thus, nine tubules were perfused with solution B, solution B plus amiloride, and solution B plus amiloride and SCH28080 designated as the basal, amiloride, and amiloride plus SCH28080 periods, respectively. The order of the periods was rotated according to balanced Latin-square design (Cochran and Cox, 1957) and there was no evidence of a time-dependent effect on K_{Rb} or V_T. As shown in Figure 4-1, in the presence of luminal Ba, 1 mM amiloride failed to stimulate K_{Rb} (81.2 ± 7.1 nm·sec^{-1}, basal period vs. 91.2 ± 10.2 nm·sec^{-1}, amiloride period) in spite of significant increase in V_T from 1.8 ± 1.5 mV during basal period to 8.5 ± 1.5 mV during the amiloride period ($P < 0.05$, Figure 4-2). The observation that Ba inhibits the stimulatory effect of amiloride on K_{Rb} indicates that a Ba-sensitive pathway mediates the effect of amiloride on Rb efflux. The parallel V_T response to amiloride regardless of the absence or presence of Ba suggests that V_T can not account for the effect of amiloride on Rb efflux. Moreover, in the presence of luminal 1 mM amiloride, SCH28080 still significantly inhibited Rb efflux from 91.2 ± 10.2 nm·sec^{-1} (amiloride period) to 68.3 ± 8.9 nm·sec^{-1} (amiloride plus SCH28080 period, $P < 0.05$, Figure 4-1). These data demonstrate that SCH28080 inhibits a pathway of Rb efflux which is unaffected by a maximum pharmacologic concentration of amiloride and insensitive to 2 mM luminal Ba. SCH28080 had no significant effect on V_T (8.5 ± 1.5 mV, amiloride period; vs 12.1 ± 2.5 mV, amiloride plus SCH28080 period, P = NS, Figure 4-2).

4.3.2 Protocol 2

To examine whether the basolateral ouabain stimulates Rb efflux which is mediated by an H-K-ATPase, seven tubules were perfused with Solution B in the absence of SCH28080 and presence of Ba. After basal K_{Rb} and V_T were measured, 0.1 mM ouabain was added to the bath. As shown in Figure 4-3, basolateral ouabain significantly increased K_{Rb} from 69.8 ± 11.1 $nm \cdot sec^{-1}$ (basal period) to 95.9± 18.7 $nm \cdot sec^{-1}$ (ouabain period, $P < 0.05$). The greater lumen-positive V_T induced by ouabain did not reach the 0.05 level of significance (-2.3 ± 2.3 mV, basal period; 8.6 ± 3.3 mV, ouabain period, $P = 0.08$, Figure 4-4)[2]. In contrast, six additional tubules were studied under identical conditions except that 10 μM SCH28080 was present in the perfusate. Perfusion with SCH28080 totally abolished the effect of ouabain on Rb efflux (69.8 ± 14.0 $nm \cdot sec^{-1}$ during basal period vs 69.6 ± 14.1 $nm \cdot sec^{-1}$ during ouabain period, P = NS, Figure 4-3), although ouabain significantly increased V_T from -1.5 ± 1.1 mV (basal period) to 2.9 ± 0.4 mV (ouabain period, $P < 0.01$, Figure 4-4). Moreover, analysis of the individual changes in V_T and changes in K_{Rb} in response to ouabain demonstrated no relationship compatible with a voltaged-mediated change in paracellular Rb efflux. In fact, the largest changes in V_T were associated with some of the smallest changes in K_{Rb}. These observations suggest that the enhancement of K_{Rb} by basolateral ouabain is mediated by H-K-ATPase.

4.3.3 Protocol 3

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[2] This lack of a significant effect of ouabain on V_T can be attributed to the fact that one tubule had a much larger voltage response to ouabain than that the other six (from -15 mV during the basal period to 27 mV during the ouabain period). Were this tubule excluded from the analysis, the effect of ouabain on voltage would be significant.

This set of experiments was designed to identify the role of a primary active K pumps in K absorption, was conducted in the absence of luminal Na, because removal of luminal Na enhances Rb efflux (Wingo and Zhou, 1990). To examine whether H-K-ATPase mediates Rb efflux, 10 μM of SCH28080 was added to the perfusate during the SCH28080 period. To examine the role of a luminal Na-K-ATPase, 0.1 mM of ouabain was added to the perfusate during the ouabain period (no SCH28080 present). Thus, nine tubules were perfused with Solution C, or Solution C plus 10 μM SCH28080, or Solution C plus 0.1 mM ouabain and these periods were designated as basal, SCH28080, and ouabain periods, respectively. The order of the periods was rotated according to balanced Latin square design (Cochran and Cox, 1957) to control for time-dependent effects. There was no evidence that the order of the periods affected either K_{Rb} or V_T (P = NS). SCH28080 significantly decreased Rb efflux by 39 ± 8.0%, from 106 ± 12.1 nm·sec^{-1} (basal period) to 65.4 ± 11.2 nm·sec^{-1} (SCH28080 period, P < 0.05, Figure 4-5). However, a maximal pharmacological dose of ouabain (0.1 mM) did not significantly affect K_{Rb} (106 ± 12.1 nm·sec-1, basal period; 84.5 ± 8.84 nm·sec-1, ouabain period, P = NS, Figure 4-5). Ouabain reduced Rb efflux only 10 ± 14% as calculated by the mean of the percent inhibition observed in the individual experiments. Vehicle experiments demonstrated that neither DMSO nor NaCl at the same concentration to that used in the experimental protocol affected Rb efflux or voltage (Table 4-2). These data demonstrate that under the present experimental conditions a luminal H-K-ATPase, not a luminal Na-K-ATPase, represents a major pathway for Ba-insensitive Rb efflux. The transepithelial voltages were not

significantly altered during this maneuver (-0.4 ± 1.1 mV, basal period, 0.1 ± 1.3 mV, SCH28080 period; and 0.4 ± 2.1 mV, ouabain period, Table 4-3).

4.4 Discussion

4.4.1 Effect of Lumen Ba

Barium is a rapidly reversible inhibitor of K channels and K conductance (Van Driessche and Zeiske, 1980). The present studies demonstrate that Ba-sensitive K permeation is present even in K-restricted rabbits. Warden et al (1989) demonstrated that Rb behaves qualitatively similar to K and concluded that there is no evidence to suggest that these ions are transported by different mechanisms. Ba-resistant Rb efflux is small in the CCD from normal rabbits (Warden et al, 1989 and Fig. 3-11), whereas in the K-restricted rabbit Ba-insensitive Rb efflux is relatively larger suggesting that K restriction increases a Ba-insensitive pathway. Since the Ba-sensitive pathway has been attributed to Ba-sensitive K channels which should mediate K secretion (Frindt and Palmer, 1989; Koeppen and Helman, 1982; Wang et al., 1990; Warden et al., 1989), the smaller Ba-sensitive pathway observed in the present study compared with that in normal rabbit is in agreement with earlier studies indicating that a reduction in K intake decreases K secretion by the CCD (O'Neil and Helman, 1977; Schwartz and Burg, 1978; Wingo et al., 1982).

4.4.2 Effect of Amiloride

It is generally accepted that both and amiloride-sensitive Na conductance and a Ba-inhibitable K conductance are present in the apical membrane of the principal cells of the CCD (Koeppen et al., 1983; Koeppen and Giebisch, 1988; Muto et al., 1988; O'Neil and Helman, 1977; Sansom et al., 1987; Sauer et al., 1990). Stokes (1984) has observed that the amiloride-induced increase in K

permeation is significantly larger than could be accounted for by the changes in voltage alone. Recently, the increment in K permeation by amiloride has been shown to be largely, via a conductive pathway in the CCD of both normal and desoxycorticosterone-treated rabbits (Warden et al., 1989). The findings that amiloride significantly increased K_{Rb} in the absence of luminal Ba (Figure 4-1), whereas amiloride failed to increase KRb in the presence of luminal Ba (Figure 4-1) are consistent with these observations. Moreover, in the presence of luminal 1 mM amiloride and 2 mM Ba, SCH28080 was still able to be demonstrated to significantly inhibit Rb efflux. These data show that SCH28080 inhibits a pathway of Rb efflux which is unaffected by a maximum pharmacologic concentration of amiloride and insensitive to 2 mM luminal Ba.

4.4.3 Effect of Peritubular Ouabain

H-K-ATPase activity may be regulated by intracellular potassium activity, and this may explain the effect of ouabain on Rb efflux. Thus, a reduction in intracellular K activity may stimulate the activity of the enzyme, whereas an increase in intracellular K activity may inhibit enzyme activity. In support of this hypothesis Koelz et al. (1981) have found that a decrease in intracellular K activity to 60 mM stimulates the gastric H-K-ATPase as measured by aminopyrine accumulation. Lorentzon et al. (1988) have demonstrated that a decrease in intracellular K activity stimulates phosphoenzyme formation and thereby increases the enzyme activity. Accordingly, maneuvers that result in a decrease in intracellular K activity should stimulate K absorption. In gastric glands intracellular K activity was significantly reduced following addition of ouabain (Koelz et al., 1981) and ouabain substantially increased K absorption in the gastric mucosa (Reenstra et al., 1986). Subsequent addition of omeprazole

significantly decreased K absorption, suggesting that the effect of ouabain on K absorption is mediated by H-K-ATPase. Ouabain significantly decreases intracellular K level of both principal cells and intercalated cells in the CCD (Sauer et al., 1989) and increases Rb efflux when SCH28080 is absent in the perfusate (Figure 4-3) whereas this maneuver fails to affect Rb efflux when SCH28080 is present in the perfusate (Figure 4-3). These data further support such a hypothesis.

The decrease in intracellular potassium concentration increasing H-K-ATPase may not unique phenomenon only obverved with peritubular ouabain. Several investigators have shown that the intracellular K level in the distal tubule was reduced by adaptation of the animals to a low K diet (Beck et al., 1982; Linas et al., 1979). Both incerase in SCH28080-sensitive ATP hydrolysis and Rb efflux were observed in the CCD of rats adapted to K-restricted diet as compared with those on a regular diet, suggesting that H-K-ATPase activity is enhanced under these conditions (Cheval et al., 1991; Doucet and Marsy, 1987).

4.4.4 Effects of Luminal SCH28080 and Ouabain

In the absence of luminal Na, luminal 10 μM SCH28080 significantly reduced K_{Rb} by 39%, whereas luminal 0.1 mM ouabain did not significantly affect K_{Rb}. These observations suggest that under the present experimental conditions, H-K-ATPase mediates in part Ba-insensitive Rb/K efflux and a ouabain-sensitive luminal Na-K-ATPase is not a major pathway for Rb/K absorption. Were an apical Na-K-ATPase mediating K absorption, the greatest degree of inhibition would be expected with luminal ouabain, whereas only the effect of SCH28080 was significant. The present demonstration is consistant with

the observations made through microenzymatic assay from which ouabain has no significant effect on ATP hydrolysis by H-K-ATPase in both rat and rabbit CCD (Doucet and Marsy, 1987; Garg and Narang, 1988). It should be emphasized that the inhibition of K absorption by ouabain has been only observed in the colonic epithelium and the distal tubule of rat and turtle urinary bladder. These observations are in contrast to the findings in the bullfrog (Ganser and Forte, 1973) and rabbit stomach (Gunther et al., 1987) and colon (Gustin et al., 1981; Halm and Frizzell, 1986) in which the H-K-ATPase is relatively resistant to inhibition by ouabain. Therefore, the species-difference should be appreciated.

4.5 Summary

In summary, amiloride increased Rb efflux, and this increment was inhibited by Ba, implying that a K-conductive pathway mediates the effect of amiloride. Basolateral ouabain increased Rb efflux in the absence of luminal SCH28080, but this same maneuver failed to affect K_{Rb} when SCH28080 was present in the perfusate, reflecting that an apical H-K-ATPase mediates the enhancement of Rb efflux following inhibition of basolateral Na-K-ATPase. Furthermore, only luminal SCH28080 not luminal ouabain was shown to inhibit Rb efflux, suggesting that a functional H-K-ATPase, not a functional Na-K-ATPase, participates in Rb efflux.

Table 4-1. Composition of solutions (in mM)

	Solution A	Solution B	Solution C
Na	135	135	
Choline			135
K	5	5	5
Cl	106.4	110.4	106.4
HCO_3	25	25	25
Ca	1.2	1.2	1.2
Ba		2	
Mg	1	1	1
Phosphate	1.5	1.5	1.5
Acetate	10	10	10
Glucose	8	8	8
Alanine	5	5	5
Mannitol	26.5	20.5	26.5
Osmal (m Osm)	324.6	324.6	324.6

Table 4-2. Effect of DMSO in the absence of luminal Na and luminal 1.5 mM

	Basal	DMSO	1.5 mM Na
K_{Rb} ($nm \bullet sec^{-1}$)	84.2 ± 18.5	82.6 ± 17.0	87.6 ± 14.9
V_T (mV)	2.8 ± 5.4	0.6 ± 2.8	1.7 ± 3.3

(n=6).

Table 4-3. Effect of luminal 10μM SCH28080 or 0.1 mM ouabain on V_T in the absence of luminal Na

	Basal	SCH28080	Ouabain
V_T (mV)	-0.4 ± 1.1	0.1 ± 1.3	0.4 ± 2.1

(n=9).

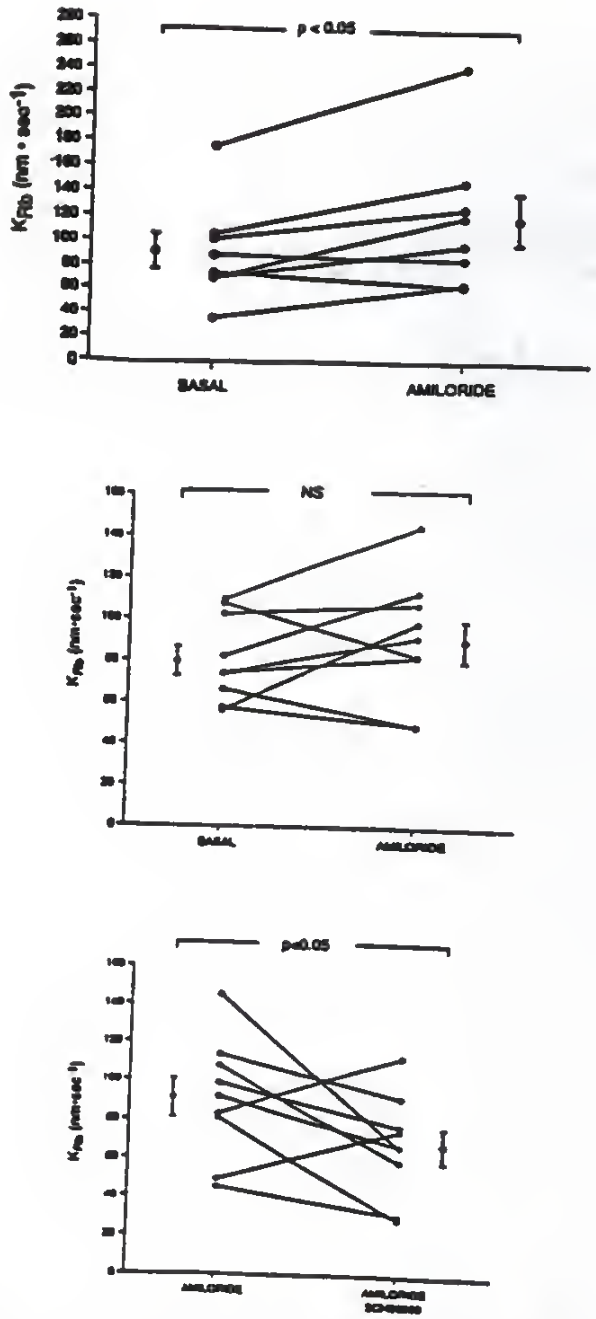

Figure 4-1. Effect of luminal 1 mM amiloride on K_{Rb} in the absence of luminal Ba (top, n = 8)and in the presence of luminal 2 mM Ba (middle, n = 9). Effect of SCH28080 on K_{Rb} in the presence of luminal 1 mM amiloride and 2 mM Ba (bottom, n = 9). Voltage-mediated paracellular K_{Rb} during amiloride periods were 15.5 ± 7.5 nm·sec^{-1} (in the absence of luminal Ba) and 25.0 ± 6.3 nm·sec^{-1} (in the presence of luminal Ba) calculated by Goldman flux equation. P_K = 1 x 10^{-5} nm·sec^{-1}. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

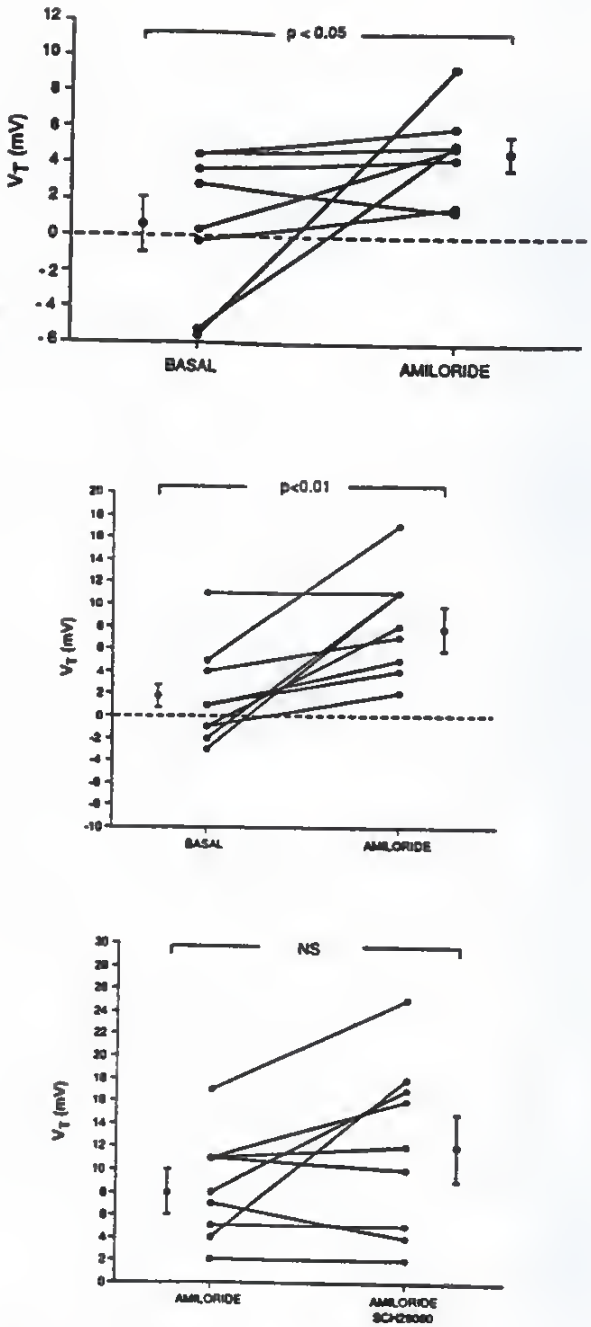

Figure 4-2. Effect of luminal 1 mM amiloride on V_T in the absence of luminal 2 mM Ba (top, n = 8) and in the presence of luminal 2 mM Ba (middle, n = 9). Effect of SCH28080 on V_T in the presence of luminal 1 mM amiloride and 2 mM Ba (bottom, n = 9). In both cases (top & middle), amiloride significantly increased VT (0.6 ± 1.5 mV vs 4.7 ± 0.9 mV, in the absence of luminal Ba; 1.8 ± 1.5 mV vs 8.5 ± 1.5 mV, in the presence of luminal Ba). Again, SCH28080 had no significant effect on V_T (8.5 ± 1.5 mV vs 12.1 ± 2.5 mV). V_T, transepithelial voltage.

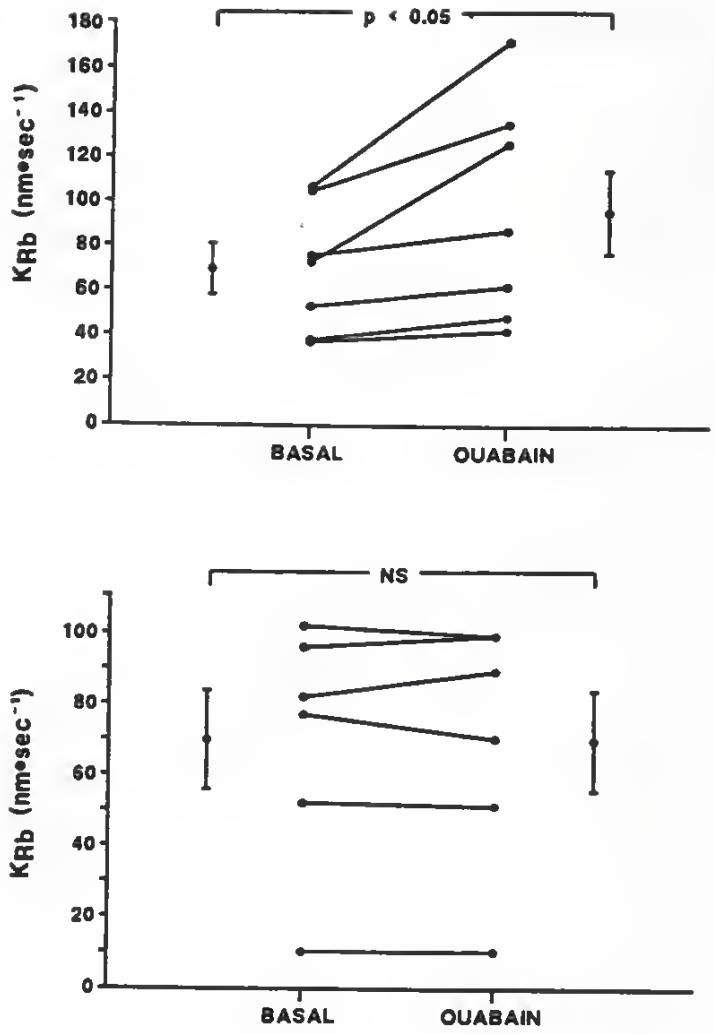

Figure 4-3. Effect of basolateral 0.1 mM ouabain on K_{Rb} in the absence of luminal SCH28080 (top, n = 7) and in the presence of luminal 10 μM SCH28080 (top, n = 6). In the absence of luminal SCH28080, ouabain significantly increased K_{Rb}, whereas in the presence of luminal SCH28080, the effect of ouabain on K_{Rb} was abolished. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

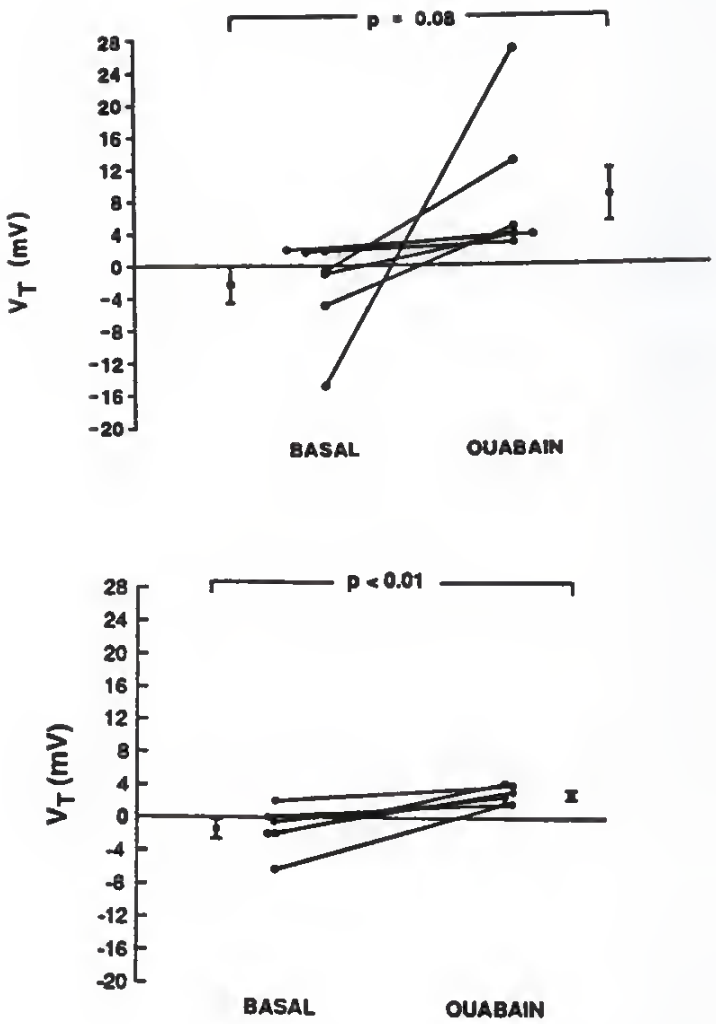

Figure 4-4. Effect of basolateral 0.1 mM ouabain on V_T in the absence of luminal SCH28080 (top, n = 7) and in the presence of luminal 10 μM SCH28080 (bottom, n = 6). Ouabain had qualitatively similar effects on V_T both in the absence and in the presence of SCH28080 although this effect was only significant at the 0.05 level in the presence of luminal SCH28080. V_T, transepithelial voltage.

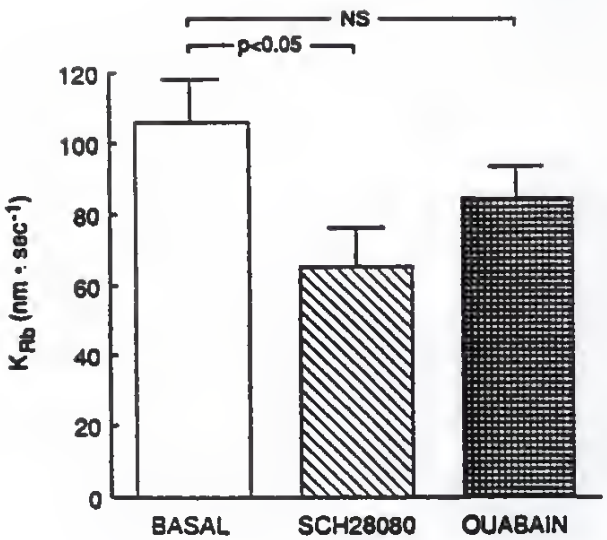

Figure 4-5. Effect of luminal SCH28080 and luminal ouabain on K_{Rb} (n=9). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

CHAPTER 5
EFFECTS OF LUMINAL SODIUM ON Rb EFFLUX

5.1 Introduction

Luminal Na removal has been demonstrated to inhibit K secretion similarly to that observed with luminal amiloride and peritubular ouabain (Stokes, 1981; Wingo, 1985). It is possible that luminal Na affects Rb efflux which is mediated by K-conductive pathway, or H-K-ATPase, or both. This hypothesis is further substantiated by the observation made in rat colon that Na removal stimulates the effect of 10% CO_2 on K absorption possibly by a closely related H-K-pump (Perron and McBride, 1988). These data suggest that luminal sodium concentration may influence H-K-ATPase activity in the CD. Therefore, the present studies aim at examination of the lumen Na removal on Rb efflux and the mechanisms underlying this effect.

5.2 Methods and Material

The tubules were dissected with Solution A (Table 5-1) containing additional 5% vol/vol feral calf serum. The bath solution which is identical to dissection solution was used throughout all sets of experiments except the study of Na efflux. All solutions were gassed to pH 7.4 with 95% O_2 and 5% CO_2. Liquid junction potentials were corrected as previously described (Wingo, 1989). The overlap of ^{86}Rb or ^{22}Na counts in the ^{3}H channel was corrected as previously described (Zhou and Wingo, 1992). SCH28080 (gift of Dr. James Kaninsky, Schering Corporation, Bloomfield, NJ) was dissolved in dimethyl

sulfoxide (DMSO). Amiloride was a gift of Merck Sharp & Dohme (Rahway, NJ) and was dissolved in the perfusate directly.

5.3 Results

5.3.1 Protocol 1

To examine whether lumen Na removal stimulates Rb efflux as lumen amiloride addition, the first set of experiments was designed to evaluate the effect of luminal Na removal on K_{Rb} in the absence of luminal Ba and the degree of inhibition of K_{Rb} by Ba in the absence of luminal Na. Six tubules were perfused with each of the following: solution A designated the basal period (135 mM Na and 0 mM Ba); solution B designated the no Na period (0 mM Na and 0 mM Ba); and solution C designated the no Na plus Ba period (0 mM Na and 2 mM Ba). The order of the periods was rotated and there was no evidence of a time dependent effect on Rb efflux. Luminal Na removal significantly stimulated K_{Rb} (Table 5-2) from 96.8 ± 22.2 $nm \cdot sec^{-1}$ (basal period) to 127 ± 20.0 $nm \cdot sec^{-1}$ (no Na period, $P < 0.005$). Luminal addition of 2 mM Ba significantly inhibited K_{Rb} from 127 ± 20.0 $nm \cdot sec^{-1}$ (no Na period) to 90.9 ± 23.0 $nm \cdot sec^{-1}$ (no Na plus Ba period, $P < 0.005$). In contrast to Rb efflux, V_T was not significantly different during the basal, the no Na, or the no Na plus Ba periods (6.3 ± 2.2 mV, 6.4 ± 1.9 mV and 7.2 ± 2.1 mV respectively, Table 5-2). The second set of experiments examined the degree of inhibition of Rb efflux by Ba when Na is present in the perfusate and the effect of luminal Na removal on K_{Rb} in the presence of luminal Ba. Six tubules were perfused with each of the following: solution A designated the basal period (135 mM Na and 0 mM Ba), solution D designated the Na plus Ba period (135 mM Na and 2 mM Ba), and solution C designated the no Na

plus Ba period (0 mM Na and 2 mM Ba). The order of the periods was rotated and there was no evidence of a time-dependent effect on K_{Rb}. Addition of 2 mM luminal Ba significantly inhibited K_{Rb} from 103 ± 17.6 $nm \cdot sec^{-1}$ (basal period) to 70.3 ± 16.6 $nm \cdot sec^{-1}$ (Na plus Ba period, $P < 0.05$, Table 5-3). Luminal Na removal significantly stimulated K_{Rb} in the presence of 2 mM luminal Ba from 70.3 ± 16.6 $nm \cdot sec^{-1}$ (Na plus Ba period), to 97.7 ± 16.6 $nm \cdot sec^{-1}$ (no Na plus Ba period, $P < 0.05$, Table 5-3). V_T was not significantly affected by Ba addition or luminal Na removal [3.1 ± 4.5 mV (basal period), -0.7 ± 3.6 mV (Na plus Ba period), and 4.7 ± 2.1 mV (No Na plus Ba period)] (Table 5-3). To determine whether K conductance was completely blocked by 2 mM Ba, we repeated the experiments under the maximal pharmacologic dose of barium which is 4 mM. To examine whether the effect of Na removal was unique only for choline substitution, sodium was replaced with TMA in this set of experiment. Thus, four tubules were perfused with Solution E (basal period) and Solution F (No Na period). The order of periods was rotated to control for time-depedent effect. There is no evidence that the order of the periods affected either Rb efflux or voltage. As shown in Table 5-4, Removal of luminal Na with TMA substitution still significantly increased Rb efflux (60.4 ± 14.4 $nm \cdot sec^{-1}$ vs 106.5 ± 14.3 $nm \cdot sec^{-1}$) without significantly affecting voltage (-9.6 ± 5.3 mV vs -5.5 ± 2.0 mV). These data indicate that stimulation of K_{Rb} following luminal Na removal is mediated in part by a Ba-insensitive pathway, and the effect of Na removal on Rb efflux is not only unique for choline substitution but also for TMA substitution.

5.3.2 Protocol 2

To examine whether H-K-ATPase mediates this enhancement in Ba-insensitive Rb efflux, we perfused the CCD in the presence of luminal 10 μM SCH28080 throughout the experiment and examined the effect of Na removal on Rb efflux. Thus, ten tubules were perfused with solution A, or solution B designated as the basal and no Na periods, respectively. The order of the periods was rotated and there was no evidence of time-dependent effect on K_{Rb} or V_T. In the presence of luminal SCH28080 (without luminal Ba), removal of luminal Na stimulated K_{Rb} from 76.7 ± 7.3 nm·sec^{-1} (basal period) to 101 ± 10.1 nm·sec^{-1} (no Na period, $P < 0.05$, Figure 5-1). Again, removal of luminal Na had no significant effect on V_T (-4.3 ± 1.3 mM, basal period; vs -1.7 ± 1.0 mM, no Na period, Figure 5-1). These data indicate that SCH28080 did not fully inhibit the increase in Rb efflux following removal of luminal Na. To examine whether both a Ba-sensitive K conductance and H-K-ATPase mediate the enhancement of Rb efflux, we examined the effect of luminal Na removal during perfusion of the CCD in the presence of both 2 mM Ba and 10 μM SCH28080. Thus, six tubules were perfused with solution D, or solution C designated as basal and no Na periods, respectively. As shown in Table 5-5, only the simultaneous presence of luminal Ba and SCH28080 inhibited the stimulation of Rb efflux by luminal Na removal (68.8 ± 9.8 nm·sec^{-1}, basal period; vs 73.3 ± 10.0 nm·sec^{-1}, no Na period, P = NS). Removal of luminal Na had no significant effect on V_T (-1.8 ± 3.2 mV, basal period, vs -0.4 ± 2.4 mV, no Na period, Table 5-5). These data indicate that both K-conductive and H-K-ATPase pathways mediate the enhancement of Rb efflux following lumen Na removal.

5.3.3 Protocol 3

To further examine whether H-K-ATPase mediates in part the increase in Rb efflux following removal of luminal Na, we examined the effect of SCH28080 both in the presence (Solution D) and in the absence (Solution C) of luminal Na with 2 mM Ba in the perfusate throughout the experiment. In each case K_{Rb} and V_T were measured in the absence of luminal SCH28080 (basal period) and in the presence of luminal SCH28080 (10 μM, SCH28080 period). The order of the periods was rotated to control for time-dependent effects. There was no evidence to suggest that the order of the periods affected Rb efflux and voltage (P = NS). As shown in Figure 5-2, in the presence of luminal Na, SCH28080 significantly reduced K_{Rb} from 78.3 ± 12.0 nm·sec^{-1} during the basal period to 67.2 ± 10.8 nm·sec^{-1} during the SCH28080 period ($P < 0.05$) without significantly affecting V_T (-2.8 ± 2.7 mV vs -1.0 ± 1.2 mV, Table 5-6). In the absence of luminal Na, SCH28080 significantly decreased K_{Rb} from 122 ± 30.3 nm·sec^{-1} to 69.4 ± 22.0 nm·sec^{-1} (Figure 5-2, $P < 0.05$) without significantly affecting V_T (5.7 ± 9.5 mV vs 6.6 ± 5.5 mV, Table 5-6). The percentage inhibition of K_{Kb} by SCH28080 was significantly less in the presence of luminal Na (14.7 ± 5.0%) as compared with the absence of luminal Na (48.2 ± 8.2%, $P < 0.01$). These experiments confirm that 1) H-K-ATPase is responsible for Rb/K absorption in the rabbit CCD, and 2) an increase in Rb/K absorption following removal of luminal Na is in part via an H-K-ATPase.

5.3.4 Protocol 4

The effect of luminal Na removal on Rb efflux is consistent with the hypothesis that Na acts as a partial agonist at the luminal K binding site and competes with K for transport via the renal H-K-ATPase. Such a hypothesis

would predict that the H-K-ATPase could transport Na when luminal K concentration is sufficiently low. Thus, we tested the effect of SCH28080 on ^{22}Na lumen-to-bath efflux coefficient (K_{Na}) when ambient K level was 0.5 mM and compared these results to similar studies when ambient K concentration was 20 mM K. To inhibit conductive Na efflux, all studies were performed in the presence of 0.1 mM luminal amiloride. Thus, in the first set of experiments, six tubules were perfused with solution G, or solution G plus 10 μM SCH28080 designated as basal and SCH28080 periods, respectively. The order of the periods was rotated and there was no evidence of time-dependent effect on K_{Na} or V_T. The second set of experiments was conducted under exactly the same conditions except for the presence of 20 mM K (perfused with solution H). In the presence of 0.5 mM K, SCH28080 significantly decreased K_{Na} from 47.6 ± 4.8 nm·sec^{-1} (basal period) to 35.0 ± 6.8 nm·sec^{-1} (SCH28080 period, $P < 0.005$, $n = 6$, Figure 5-3). In contrast, in the presence of 20 mM K, SCH28080 had no significant effect on K_{Na} (33.3 ± 5.0 nm·sec^{-1}, basal period; vs 30.6 ± 5.2 nm·sec^{-1}, SCH28080 period, P = NS, $n = 8$, Figure 5-3). Because SCH28080 has ben shown to compete with K at K binding site of gastric H-K-ATPase, and high K concentration abolishes the inhibitory effect of SCH28080 on gastric H-K-ATPase (Keeling et al, 1988; Mendlein and Sachs, 1990), to identify that the lack of inhibition of K_{Na} by SCH28080 in the presence of 20 mM K is due to potassium diminishing Na efflux or due to K demolishing the inhibitory effect of SCH28080, the third set of experiments was designed to examine the effect of SCH28080 on Rb efflux in the presence of 20 mM K. Thus, eight tubules were perfused with Solution F. As shown in Table 5-7, SCH28080 significantly reduced K_{Rb} from 97.9 + 12.0 nm.sec^{-1} (basal period)

to 79.2 + 9.2 nm.sec^{-1} (SCH28080 period). These data demonstrate that Na can be transported via the renal H-K-ATPase by competing with K. In either of the cases, SCH28080 had no significant effect on V_T (in the first set, 0.5 mM K, 16.7 ± 5.2 mV, basal period; vs 18.1 ± 6.3 mV, SCH28080 period, Figure 5-4, and in the second set, 20 mM K, 5.9 ± 0.7 mV basal period; vs 6.4 ± 0.8 mV, SCH28080 period, Figure 5-4, in the third set, 6.0 + 1.5 mV, basal period; vs 5.8 + 1.5 mV SCH28080 period, Table 5-7), suggesting that 10 μM SCH28080 had no significant effect on an electrogenic process.

5.4 Discussion

5.4.1 Effect of Lumen Na Concentration

The application of 2 mM luminal Ba produced significant inhibition of Rb efflux regardless of the presence or absence of luminal Na (Table 5-2 and 5-3). Removal of luminal Na produced a highly significantly increase in K_{Rb} when Ba was absent in the lumen (Table 5-2), and this effect can not be blocked by either Ba (Table 5-3) or SCH28080 (Figure 5-1) alone. Only the combined application of Ba and SCH28080 in the perfusate inhibited the stimulatory effect of Na removal (Table 5-5), suggesting that both a Ba-sensitive K conductance and H-K-ATPase mediate the enhancement of Rb efflux following removal of luminal Na, at least during K restriction. Such a hypothesis is further substantiated by the subsequent observations. When the CCD was perfused with 2 mM Ba, SCH28080 produced a greater degree of inhibition of K_{Rb} in the absence of luminal Na than in the presence of luminal Na. These results imply that Rb efflux is stimulated in part via an H-K-ATPase following removal of luminal Na. On the other hand, 2 mM Ba inhibited the stimulatory effect of amiloride on K_{Rb} (Figure 4-1), suggesting that only a Ba-sensitive K

pathway mediates the effect of amiloride. These data provide clear evidence that lumen Na removal has more generalized effect on Rb efflux than lumen amiloride addition. Several investigators have shown that removal of Na results in a more lumen-positive V_T (O'Neil and Boulpaep, 1979), whereas addition of Ba makes V_T more lumen-negative (Frindt and Palmer, 1989; O'Neil, 1985). However, appreciable transepithelial voltage changes were not observed in the present study. The discrepancy may be due to the different measurement of V_T and the different diets used in the present studies. In the previous experiments, the instantaneous voltage changes following removal of Na (O'Neil and Boulpaep, 1979) and addition of Ba (Frindt and Palmer, 1989; O'Neil, 1985) were monitored, whereas in the present study, we measured the average voltage over the time of the sample collection. Equally important, all previous experiments were conducted on K-replete animals (O'Neil and Boulpaep, 1979; Frindt and Palmer, 1989; O'Neil, 1985), whereas the present results were performed in K-restricted rabbits. The plasma aldosterone level is reduced during K-restriction (Wingo, 1987) and it is generally accepted that K intake and mineralocorticoid levels have significant effects on Na and K transport (Muto et al, 1988; O'Neil and Helman, 1977; Sansom and O'Neil, 1985 & 1986; Schwartz and Burg, 1978; Wingo et al., 1982). Moreover, removal of luminal Na does not significantly affect V_T in the CCD from adrenalectomized rabbits (Wingo, 1985). Thus, one explanation for the lack of an effect of luminal Na removal on V_T in the present experiments is the significantly reduced mineralocorticoid activity.

5.4.2 Interaction Between Na and K

The stimulatory effect of luminal Na removal on Rb efflux via H-K-ATPase may be similar to the mechanism proposed for a closely related K-ATPase in the colonic epithelium. Although the pharmacologic characteristics of this putative pump were not characteristic of the gastric H-K-ATPase, Sweiry and Binder (1990) demonstrated that Na absorptive flux, like K-absorptive flux, was inhibited by orthovanadate and mucosal ouabain when mucosal and serosal K concentration was 0.6 mM. However, when K level was 5.2 mM in the mucosal and serosal solutions, both drugs had less effect on Na absorption. The authors interpreted the data as evidence that mucosal Na competed with mucosal K for uptake across the apical membrane. Although the putative pump described by Sweiry and Binder was relatively resistant to SCH28080 but sensitive to mucosal ouabain, it should be noted that a similar K-absorptive mechanism in the rabbit colon is not inhibited by ouabain (Gustin and Goodman, 1981; Halm and Frizzell, 1986). Thus species-specific differences in the pharmacologic sensitivity of K-absorbing pumps may be more important than previously appreciated. In support of such a hypothesis, SCH28080 significantly inhibited Na efflux when the tubules were perfused in the presence of 0.5 mM K (Figure 5-3), whereas the effect of SCH28080 was not observed when the tubules were perfused in the presence of 20 mM K (Figure 5-3). Because SCH28080 has ben shown to compete with K at K binding site of gastric H-K-ATPase, and high K concentration abolishes the inhibitory effect of SCH28080 on gastric H-K-ATPase (Keeling et al., 1988; Mendlein and Sachs, 1990), to determine whether the lack of inhibition of K_{Na} by SCH28080 in the presence of 20 mM K is due to potassium diminishing Na efflux or due to K demolishing the inhibitory effect of SCH28080, we examined the effect of

SCH28080 on Rb efflux in the presence of 20 mM K. The results from this set of experiments suggests that the lack of effect of SCH28080 on Na efflux in the presence of 20 mM K is not due to the lack of inhibitory effect of SCH28080 on H-K-ATPase (Table 5-7). These data suggest that Na competes with K for absorption via the H-K-ATPase. In contrast, microenzymatic assay has shown that ATP hydrolysis by this enzyme is not Na-dependent (Doucet and Marsy, 1987; Garg and Narang, 1988). The different results between the previous studies and present studies may be reconciled by different measurement. In the previous studies ATP hydrolysis was detected in the permeabolized tubules in the absence of K, whereas in the present studies Rb efflux was measured in the intact tubules. It is not unlikely that H-K-ATPase need certain amount of K to maintain its activity. More importantly, Na entry to the cell is down its electrochemical potential. H-K-ATPase may operate in a fashion similar to Na-H antiport when this enzyme transports Na. In this case, H-K-ATPase operates like a secondary active transporter and may not need ATP hydrolysis to supply energy. Moreover, SCH28080 was still able to inhibit Rb efflux even in the presence of a maximum pharmacologic dose of amiloride (Figure 4-1) demonstrating that H-K-ATPase is pharmacologically distinguishable from a Na-H antiporter.

5.5 Summary

The increase in Rb efflux by luminal Na removal was only fully blocked by the simultaneous presence of luminal Ba and SCH28080 indicating that both Ba-sensitive K conductance and H-K-ATPase mediate the effect of lumen Na removal. The potential clinical significance of the present observations includes an additional mechanism to explain the impairment in net K secretion when

distal Na delivery is reduced. A decrease of net K secretion following reduction in luminal sodium concentration (Stokes, 1981; Wingo, 1985) may reflect in part enhanced K-absorptive flux (lumen-to-blood) as well as decreased K secretory flux (blood-to-lumen). Indeed, in K-depleted animals, the delivery of Na to the distal tubule and collecting duct is markedly reduced (Khuri et al., 1975; Walter et al., 1988). This would provide a better environment for H-K-ATPase to function optimally in order to conserve K whereas an increase in distal Na delivery would promote a kaliuresis (Tannen and Gerrits, 1986). The presence of SCH28080-sensitive Na efflux is reduced by increasing ambient K concentration suggesting that Na competes with K for transport via the H-K-ATPase. These observations may have direct relevance to the observed Na retension that accompanies severe K depletion. Furthermore, the inhibition of K_{Rb} by SCH28080 in the presence of 1 mM amiloride indicates that an H-K-ATPase is pharmacologically distinguishable from a Na-H exchanger.

Table 5-1. Composition of solutions (in mM)

	Solution A	Solution B	Solution C	Solution D	Solution E	Solution F
Na	135			135	135	
Choline		135	135			
K	5	5	5	5	5	5
Cl	106.4	106.4	110.4	110.4	115.4	114.4
HCO_3	25	25	25	25	25	25
Ca	1.2	1.2	1.2	1.2	1.2	1.2
Ba			2	2	4	4
Mg	1	1	1	1	1	1
Phosphate	1.5	1.5	1.5	1.5		
Acetate	10	10	10	10	10	10
Glucose	8	8	8	8	8	8
Alanine	5	5	5	5	5	5
Mannitol	26.5	26.5	26.5	26.5	4.5	7.5
TMA						132
Osmolarity (mOsm)	324.6	324.6	324.6	324.6	313.3	313.3

Table 5-1. Composition of solutions (in mM, continue)

	Solution G	Solution H
Na	28	28
Choline	102	82.5
K	0.5	20
Cl	106.9	106.9
HCO_3	25	25
Ca	1.2	1.2
Mg	1	1
Phosphate	1.5	1.5
Acetate	10	10
Glucose	8	8
Alanine	5	5
Mannitol	26.5	26.5
Osmolarity (mOsm)	298.6	298.6

Table 5-2. Effect of removal of luminal Na in the absence of luminal Ba, and of luminal Ba addition in the absence of luminal Na

	Basal (135 mM Na 0mMBa)	No Na (0 mM Na 0mM Ba)	No Na + Ba (0mM Na 2mM Ba)
K_{Rb} ($nm \cdot sec^{-1}$)	96.8 ± 22.2	127 ± 20.0[a]	90.9 ± 23.0[b]
V_T (mV)	6.3 ± 2.2	6.4 ± 1.9	7.2 ± 2.1

[a] $p<0.005$, compared with basal period. [b] $p<0.005$, compared with no Na period ($n=6$). Tubules were perfused with Solution A (basal period), Solution B (no Na period) and Solution C (no Na plus Ba period). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transepithelial voltage.

Table 5-3. Effect of luminal Ba addition in the presence of luminal Na, and of luminal Na removal in the presence of luminal Ba

	Basal (135 mM Na 0mMBa)	Na plus Ba (135 mM Na 2mM Ba)	No Na + Ba (0 mM Na 2mM Ba)
K_{Rb} ($nm \cdot sec^{-1}$)	103 ± 17.6	70.3 ± 16.6[a]	97.7 ± 16.6[b]
V_T (mV)	3.1 ± 4.5	-0.7 ± 3.6	4.7 ± 2.1

[a] $p<0.05$, compared with basal period. [b] $p<0.05$, compared with Na plus Ba period (n=6). Tubules were perfused with Solution A (basal period), Solution D (Na plus Ba period) and Solution C (no Na plus Ba period). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transepithelial voltage.

absence of luminal Na

	Basal (135 mM Na 0mMBa)	No Na (0 mM Na 0mM Ba)	No Na + Ba (0mM Na 2mM Ba)
K_{Rb} ($nm \cdot sec^{-1}$)	96.8 ± 22.2	127 ± 20.0[a]	90.9 ± 23.0[b]
V_T (mV)	6.3 ± 2.2	6.4 ± 1.9	7.2 ± 2.1

[a] $p<0.005$, compared with basal period. [b] $p<0.005$, compared with no Na period ($n=6$). Tubules were perfused with Solution A (basal period), Solution B (no Na period) and Solution C (no Na plus Ba period). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transepithelial voltage.

Table 3-3. Effect of luminal Ba addition in the presence of luminal Na, and of luminal Na removal in the presence of luminal Ba

	Basal (135 mM Na 0mMBa)	Na plus Ba (135 mM Na 2mM Ba)	No Na + Ba (0 mM Na 2mM Ba)
K_{Rb} ($nm \cdot sec^{-1}$)	103 ± 17.6	70.3 ± 16.6[a]	97.7 ± 16.6[b]
V_T (mV)	3.1 ± 4.5	-0.7 ± 3.6	4.7 ± 2.1

[a] $p<0.05$, compared with basal period. [b] $p<0.05$, compared with Na plus Ba period (n=6). Tubules were perfused with Solution A (basal period), Solution D (Na plus Ba period) and Solution C (no Na plus Ba period). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transepithelial voltage.

Table 5-4. Effect of removal of luminal Na in the presence of 4 mM Ba

	Basal	No Na
K_{Rb} ($nm \cdot sec^{-1}$)	60.4 ± 14.4	106.5 ± 14.3*
V_T (mV)	-9.6 ± 5.3	-5.5 ± 2.0

K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transpithelial voltage (n=4).
* $p < 0.005$.

Table 5-5. Effect of luminal Na removal in the presence of both 2 mM Ba and 10 μM SCH28080

	Basal	No Na
K_{Rb} ($nm \cdot sec^{-1}$)	68.8 ± 9.8	73.3 ± 10.0
V_T (mV)	-1.8 ± 3.2	-0.3 ± 2.4

K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient; V_T, transpithelial voltage (n=6).

Table 5-6. Effect of luminal SCH28080 on V_T in the absence and presence of luminal Na

	135 mM Na	
	Basal	SCH28080
V_T (mV) (n=6)	-2.8 ± 2.7	-1.0 ± 1.2
	No Na	
	Basal	SCH28080
V_T (mV) (n=4)	-5.7 ± 9.5	-6.6 ± 5.5

Table 5-7. Effect of SCH28080 in the presence of 20 mM K

	Basal	SCH28080
K_{Rb} ($nm \bullet sec^{-1}$)	97.9 ± 12.0	79.2 ± 9.2
V_T (mV)	6.0 ± 1.5	5.8 ± 1.5

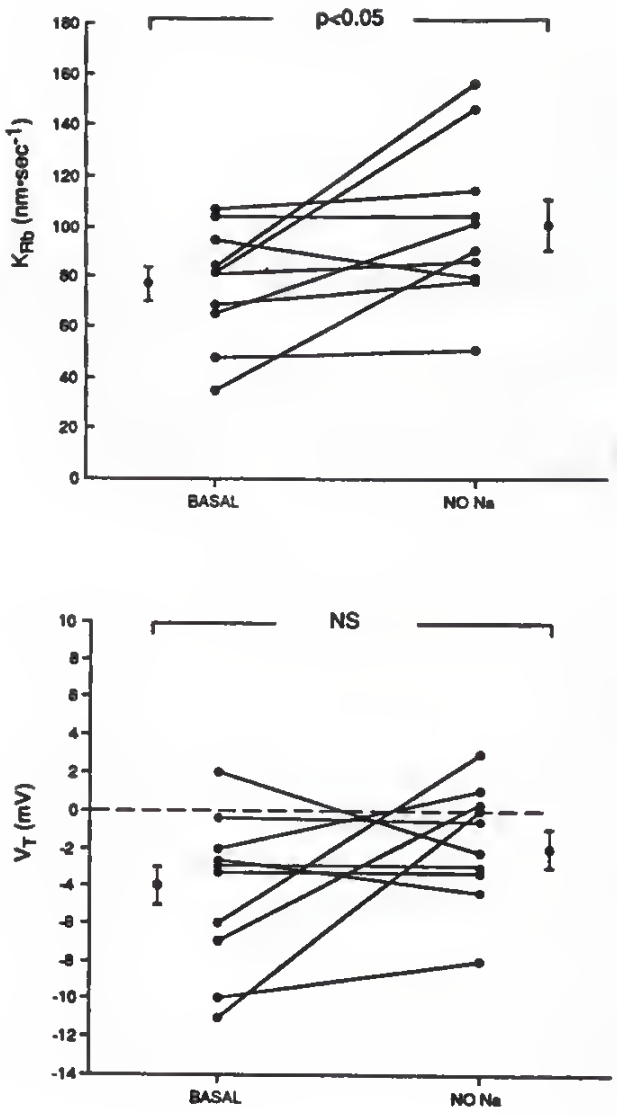

Figure 5-1. Effect of lumen Na removal in the presence of luminal 10 μM SCH28080 on K_{Rb} (top) and on V_T (bottom, n = 10). Removal of luminal Na significantly stimulated K_{Rb} from 76.7 ± 7.3 nm·sec^{-1} to 101 ± 10.1 nm·sec^{-1} but had no significant effect on V_T (-4.3 ± 1.3 mV vs -1.7 ± 1.0 mV). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

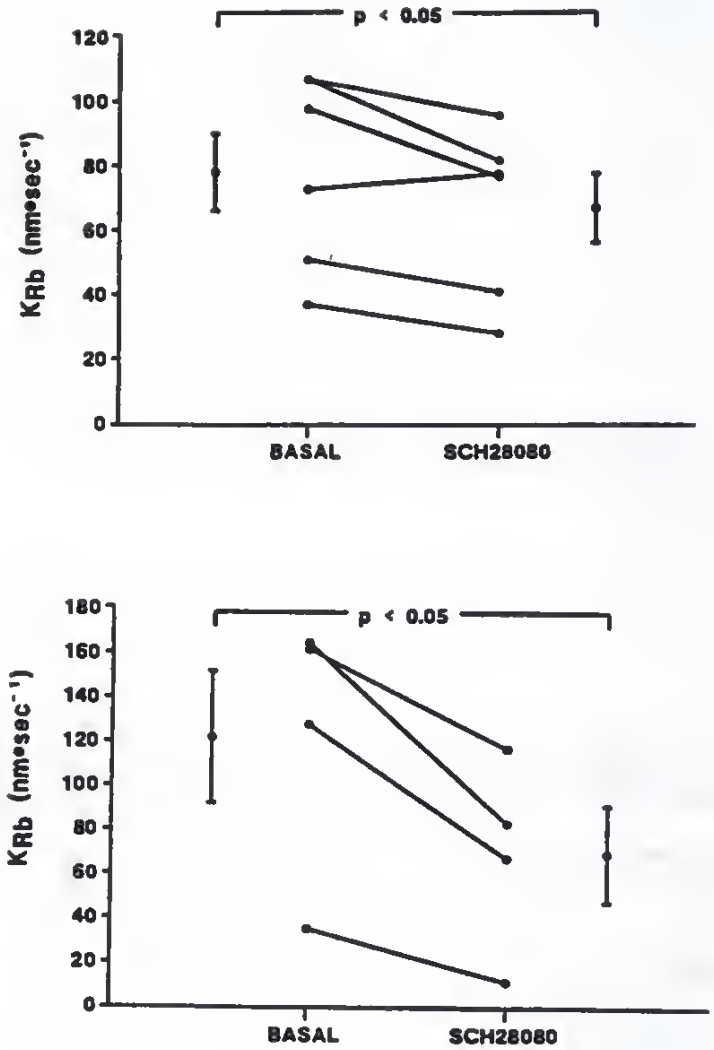

Figure 5-2. Inhibition of K_{Rb} by luminal 10 μM SCH28080 in the presence of luminal Na (top, n=6) and in the absence of luminal Na (bottom, n = 4). In both cases SCH28080 significantly inhibited K_{Rb}. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

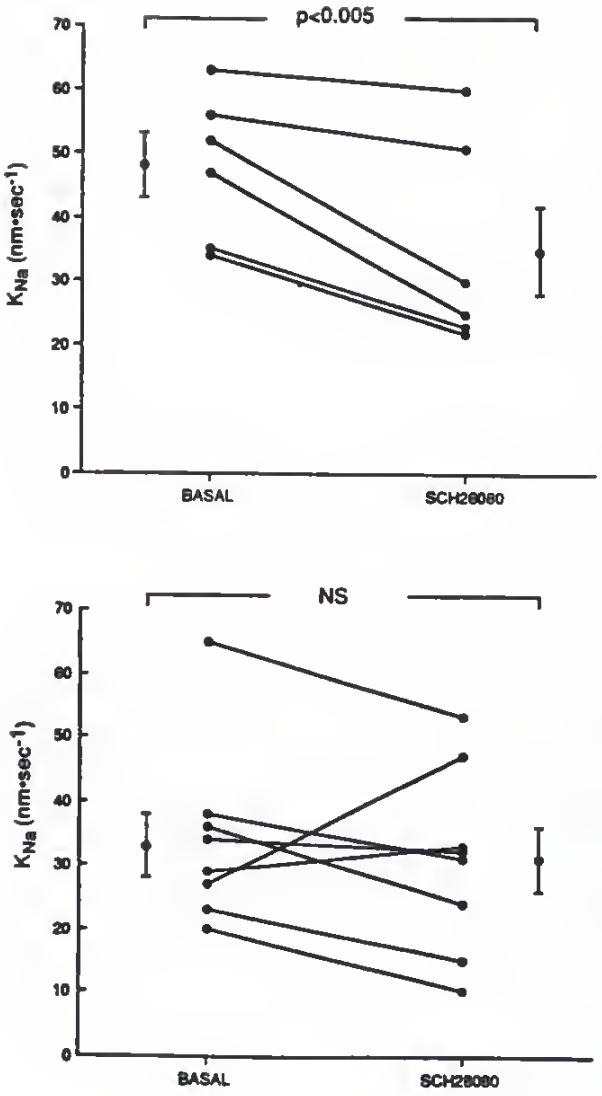

Figure 5-3. Effect of SCH28080 on K_{Na} in the presence of ambient 0.5 mM K (top) and in the presence of ambient 20 mM K (bottom). In the presence of 0.5 mM K, SCH28080 significantly decreased K_{Na} from 47.6 ± 4.8 nm·sec^{-1} to 35.0 ± 6.8 nm·sec^{-1} (n = 6), whereas in the presence of 20 mM K SCH28080 failed to inhibit K_{Na} (33.3 ± 5.0 nm·sec^{-1} vs 30.6 ± 5.2 nm·sec^{-1}, n = 8). K_{Na}, ^{22}Na lumen-to-bath efflux coefficient.

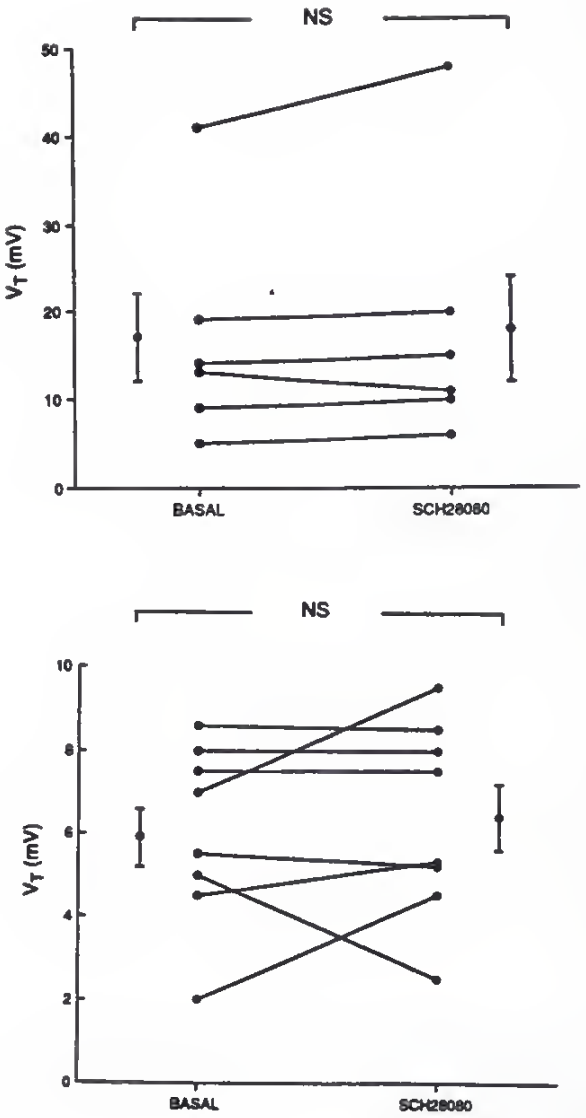

Figure 5-4. Effect of SCH28080 on V_T in the presence of ambient 0.5 mM K (top, n = 6) and in the presence of ambient 20 mM K (bottom, n = 8). In either of cases SCH28080 had no significant effect on V_T (16.7 ± 5.2 mV vs 18.1 ± 6.3 mV, in the presence of ambient 0.5 mM K; 5.9 ± 0.7 mV vs 6.4 ± 0.8 mV, in the presence of ambient 20 mM K). V_T, transepithelial voltage.

CHAPTER 6
EFFECT OF ANGIOTENSIN II, HISTAMINE, AND CARBACHOL

6.1 Introduction

Angiotensin II is a potent stimulator of proton secretion and solute transport by the proximal tubule (Harris and Navar, 1985; Liu and Cogan, 1987; Mujais et al., 1986; Schuster et al., 1984). Mujaris et al. (1986) have demonstrated specific angiotensin II binding in the collecting duct, yet a functional role for this hormone in the collecting duct has not been examined. Angiotensin II has been shown to be antikaliuretic in human when infused at the concentrations that do not elicit systemic or renal hemodynamic changes (McMurray and Struthers, 1988). This observation is consistant with hypothesis that Ang II stimulate H-K-ATPase. Alternatively, Ang II may inhibit K secretion. Therefore, The first studies aim at identification of whether Ang II increases H-K-ATPase activity, or decreases K secretion, or both.

The renal H-K-ATPase has been demonstrated to be pharmacologically (Wingo, 1989), or even molecular biologically similar to the gastric H-K-ATPase (Okusa et al., 1990). Because histamine and carbachol substantially stimulate the gastric H-K-ATPase (Forte et al., 1981; Koelz et al., 1981), the second studies aim at examination of whether these two hormones exhibit the similar effect on renal H-K-ATPase.

6.2 Methods and Material

The tubules were dissected with Solution A (Table 6-1) containing additional 5% vol/vol fetal calf serum. Three bathes and perfusates were used in these studies as approriate. All of solutions were gased to pH 7.4 with 5% CO_2 and 95% O_2. Angiotensin II, histamine and carbachol were dissolved in 0.9% NaCl solution and applied to bath with the final concentrations of 0.1 nM, 10 μM, and 0.1 mM, respectively.

6.3 Results

6.3.1 Protocol 1

To examine whether Ang II enhances Rb efflux, the first set of experiments was performed in the absence of luminal SCH28080. To eliminate possible inhibition of K conductance by Ang II which might overshadow the stimulatory effect of Ang II on Rb efflux, 3 mM Ba was present in the perfusate. Three tubules were perfused with Solution A as bath, the other three were perfused with same solution except for absence of phosphate. Thus, six tubules were perfused with Solution B (Table 6-1) as the perfusate and Solution A as the bath. Under these conditions, 0.1 nM angiotensin II failed to stimulate Rb efflux (64.5 ± 11.2 $nm.sec^{-1}$ vs 54.5 ± 10.5 $nm.sec^{-1}$, n=6, Fig. 6-1), suggesting that angiotensin II has no significant effect on K absorptive flux. Ang II significantly increased lumen-negative voltage (-0.7 ± 2.9 mV vs -10.4 ± 3.8 mV, n=6, $p<0.01$, Fig. 6-1). Alternatively, to examine whether Ang II inhibits K conductance, the second set of experiments was conducted in the absence of luminal Ba and presence of luminal SCH28080 with Solution A as perfusate and bath, 0.1 nM Ang II significantly reduced Rb efflux (97.7 ± 16.5 $nm.sec^{-1}$ vs 72.4 ± 14.3 $nm.sec^{-1}$, $p<0.05$, n=6, Fig. 6-2). The voltage became more lumen-negative during angiotensin II period (0.9 ± 2.6 mV vs -7.0 ± 2.7 mV,

$p<0.05$, n=6, Fig. 6-2). Taken together, this suggests that inhibition of Rb efflux by Ang II depends on the Ba-sensitive K conductive pathway.

6.3.2 Protocol 2

To examine whether histamine stimulates Rb efflux, the experiments were performed in the absence of luminal SCH28080 and presence of luminal 3 mM Ba with Solution A as bath and Solution B as perfusate. 10 μM histamine had no significant effect on Rb efflux, and the observation from 30 to 90 minutes later after the removal of histamine indicates that perfusion time did not affect Rb efflux (77.9 ± 13.8 $nm.sec^{-1}$, basal period; 79.5 ± 16.8 $nm.sec^{-1}$, histamine period; 73.3 ± 20.9 $nm.sec^{-1}$, 30-60 minutes after histamine; and 80.9 ± 20.8 $nm.sec^{-1}$, 60 to 90 minutes after histamine, n=5, Fig. 6-3). The voltage has not been significantly changed by these maneuvers (-11.8 ± 5.8 mV, basal period; -11.6 ± 7.4 mV, histamine period; -9.2 ± 7.5 mV, 30 to 60 minutes after histamine; and -7.6 + 6.3 mV, 60 to 90 minutes after histamine, n=5, Table 6-2). This suggests that histamine did not stimulate H-K-ATPase under the present experimental conditions. Carbachol has been shown to cause natriuresis. Inhibition of Na transport by removal of luminal Na and addition of peritubular ouabain has been demonstrated to stimulate Rb efflux mediated by H-K-ATPase. The next set of experiments was designed to examine whether carbachol affects both Na and K transport with Solution C as bath and perfusate). For the technical convenience, the net chemical flux was measured using flameless atomic absorption spectrophotometer during this study. As shown in Table 6-3, although carbachol significantly stimulated Na absorptive flux from 5.0 ± 2.2 pmol/mm/min to 11.5 ± 2.3 pmol/mm/min, the magnitude of this stimulation is quite small and has no physiological significance. The effect of

carbachol on K flux was not significant (-3.1 ± 0.4 pmol/mm/min vs -1.6 ± 0.5 pmol/mm/min), suggesting that carbachol has no significant effect on H-K-ATPase.

6.4 Discussion

6.4.1 Effect of Angiotensin II

In human subject, infusion of angiotensin II cause antikaliuretic at the concentration which does not elicit systemic and renal hemodinamic changes (McMurray and Struthers, 1988). This effect may be attributed to increase K absorption, or inhibit K secretion, or both. The present studies have shown that Ang II at the physiological concentration has no significant effect on Rb efflux, indicating that Ang II has little effect on K absorptive process. On the other hand, Ang II significantly inhibited Rb efflux which was mediated by K-conductive pathway. The present results are compatible with other studies made at cellular level. Angiotensin II has been shown to activate protein kinase C and increase intracellular Ca activity. Wang et al. (Wang et al., 1991; Wang and Giebisch, 1990) have provided the evidence that protein kinase C and intracellular Ca decrease the open probability of the low conductance of K channel of the collecting duct from their patching-clamp studies. Because this type of K-conductance (Frindt and Palmer, 1989; Wang et al., 1990), not Ca-activated K channel (Frindt and Palmer, 1987; Hunter et al., 1986), has been proposed to be a strong candidate for K secretion, This suggests that Ang II inhibit K secretion. In addition, Brauneis et al. (1991) have recently demonstrated that angiotensin II blocks outward potassium currents in zona glomerulosa cells from rat, bovine, and human adrenals using whole cell patch clamp. With use of cell-attached mode, single-channel recordings in bovine zona

glomerulosa cells have shown that K channels are reversibly blocked when Ang II was added to the bath solution. These observations are compatible with the results of the present studies.

6.4.2 Effect of Histamine and Carbachol

The present data do not support the positive role of histamine and carbachol in stimulation of renal H-K-ATPase, although these two hormones are the potent stimulators of gastric H-K-ATPase (Forte et al, 1981; Koelz et al, 1981). Many possibilities could be proposed to explain these observations. However, either no receptors of the hormones in the CCD or the different signal transduction of the renal H-K-ATPase from the gastric H-K-ATPase is most plausible.

6.5 Summary

Angiotensin II inhibited Rb efflux mediated by a Ba-sensitive conductance, suggesting that Ang II inhibit K secretion. The stimulatory effect of histamine and carbachol on Rb efflux or K absorptive flux was not observed in the present studies, implying that under the present conditions these hormones do not stimulate H-K-ATPase.

Table 6-1. The composition of solutions (in mM).

	Solution A	Solution B	Solution C
Na	138	135	145
K	5	5	5
Cl	109.4	115.4	112
HCO_3	25	25	25
Ca	1.2	1.2	1.8
Mg	1	1	1
Acetate	10	10	10
Glucose	8	8	8
Alanine	5	5	5
Ba		3	
Phosphate	1.5		2.3
SO_4			1
Mannitol	9	4.5	
Osmol (m Osm)	313.1	313.1	316.1

Table 2. Effect of histamine on V_T in the presence of luminal 3mM Ba

	Basal	Histamine	Recovery (30-60 min)	Recovery (60-90 min)
V_T (mV)	-11.8 ± 5.8	-11.6 ± 7.4	-9.2 ± 7.5	-7.6 ± 6.3

Table 6-3. Effect of carbachol

	Basal	Carbachol
J_{Na} (pmol/mm/min)	5.0 ± 2.2	11.5 ± 2.3*
J_K (pmol/mm/min)	-3.1 ± 0.4	-1.6 ± 0.5
V_T (mV)	1.4 ± 1.5	1.3 ± 1.7

* $p<0.05$, vs the basal period (n=6).

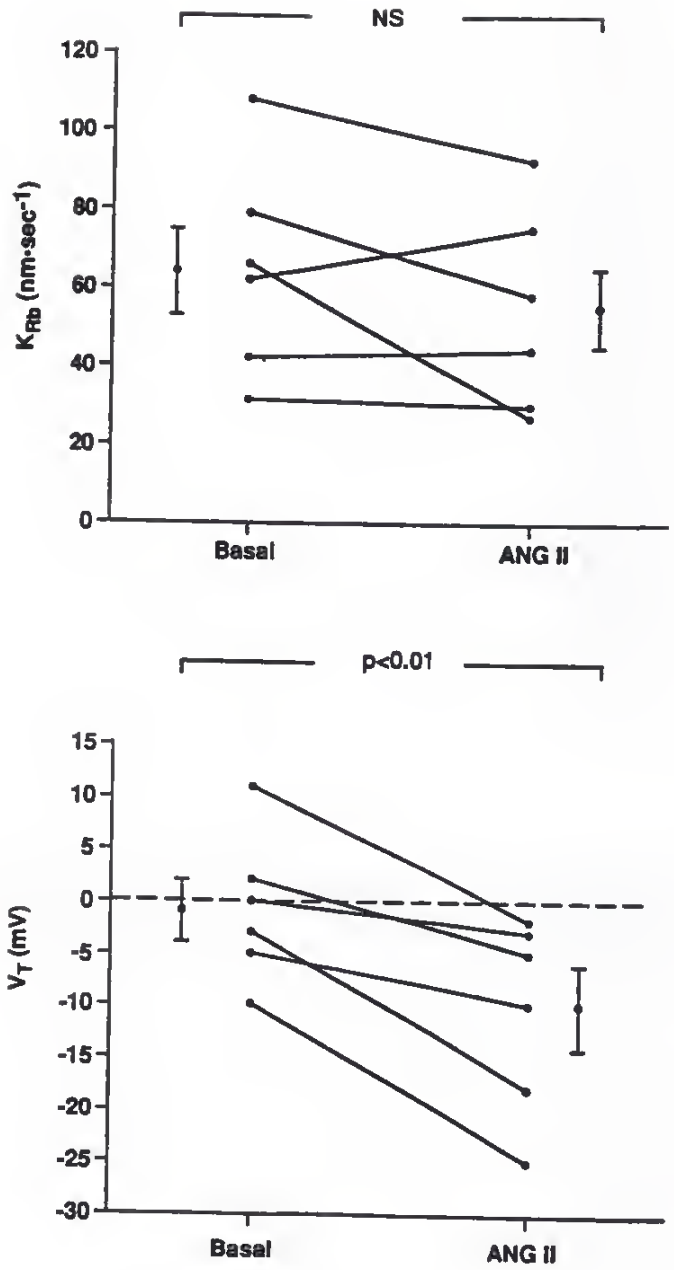

Figure 6-1. Effect of angiotensin II on K_{Rb} (top) and V_T (bottom, n=6) in the presence of luminal 3mM Ba and absence of luminal SCH28080. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

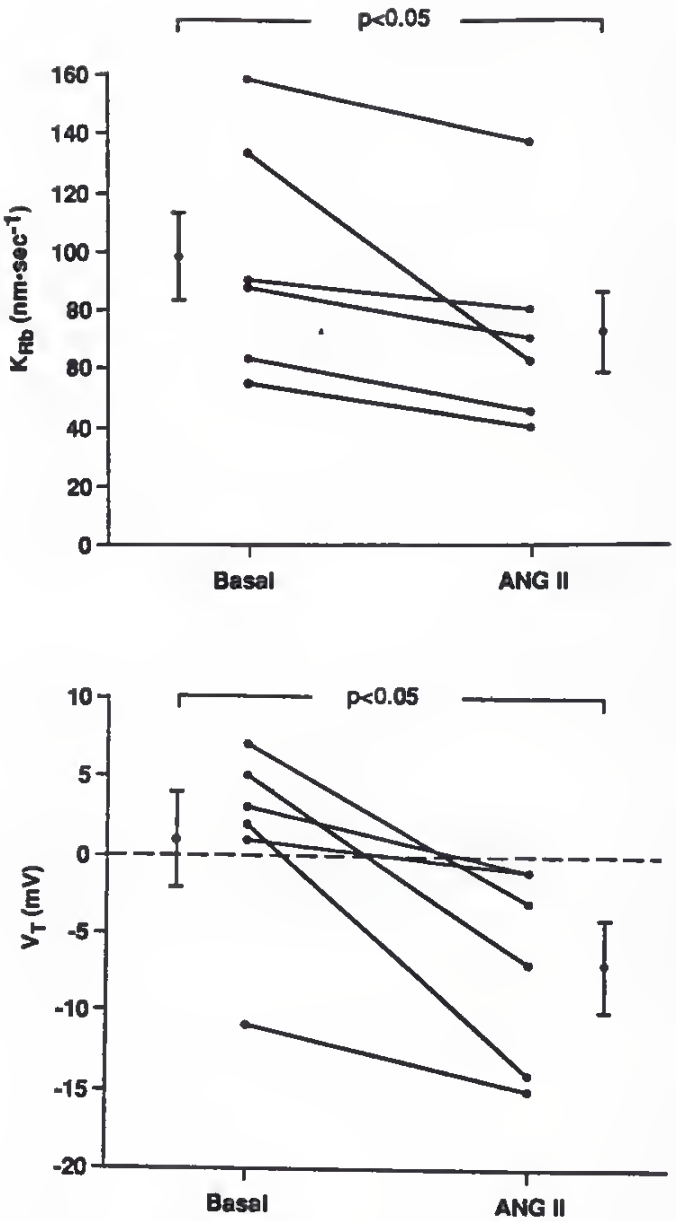

Figure 6-2. Effect of angiotensin II on K_{Rb} (top) and V_T (bottom, n=6) in the presence of luminal 10μM SCH28080 and absence of luminal Ba. K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient. V_T, transepithelial voltage.

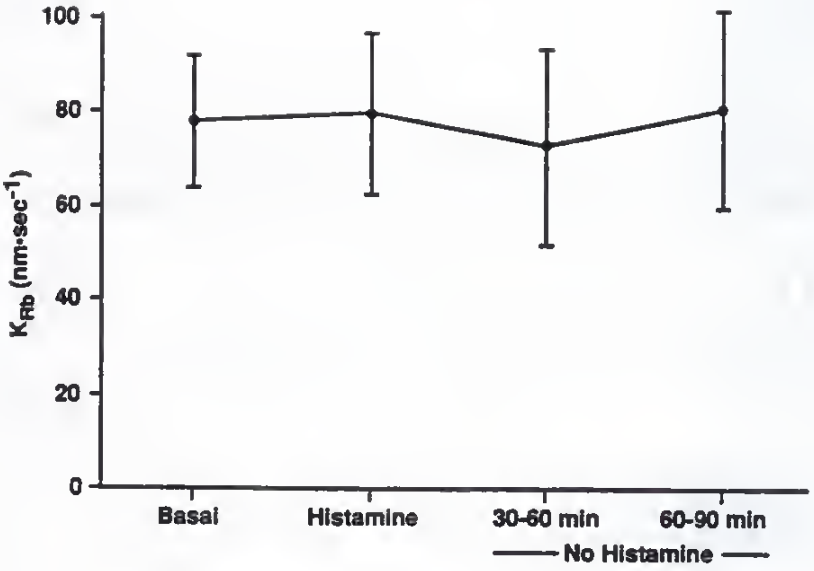

Figure 6-3. Effect of histamine on K_{Rb} in the presence of luminal 3mM Ba (n=5). K_{Rb}, ^{86}Rb lumen-to-bath efflux coefficient.

CHAPTER 7
EFFECT OF Na AND K INTAKE ON SERUM AND URINE Na AND K LEVELS AND URINE OUTPUT

7.1 Introduction

It has been widely documented that low K and low Na intakes decrease urinary output of Na and K, plasma level of K, and disturb acid-base balance. To examine whether these observations arers

reproducible in our system, we conducted the metabolic studies.

7.2 Methods and Material

Two different diets (TD 87317 and TD 87433, Teklad, Madison, WI) were used as appropriate. Blood was drawn from central ear artery. Urinary sample was collected as appropriate. The concentrations of sodium and potassium were measured by the auto-analyzer (ASTRA 880, Beckman, Somerset, NJ).

7.3 Results

7.3.1 Protocol 1

To examine whether restrictions of Na and K intake decrease plasma level and urinary excretion of Na and K and increase urine output, the rabbits were adapted to a low Na (0% Na) and low K (0.25% K) diet (TD 87317) for nine days. Urinary Na and K level and urine output were measured each day. The plasma levels of Na and K were measured on 9th day of adaptation to this diet. As shown in Table 7-1, there was a tendancy in decrease in urinary

Na level one day after the animals were exposed to the diet. Urinary Na concentration was significantly reduced from 2nd day on the diet (at least $p<0.05$), and no further reduction in Na level was observed for the entire period on the diet. However, the rabbits excreted K differently from Na. A significant reduction in urinary K level (at least $p<0.01$) was observed one day after the animals were on the diet, and a further reduction in K concentration (at least $p<0.05$) was observed four days after the rabbits were on the diet. The total urinary excretion of Na and K was significantly reduced one day after the diet (at least $p<0.01$). The total excreted Na and K were further reduced eight days after the diet because the urine output decreased. Moreover, there was a trend in increase in urine output after animals were adapted to the diet. However, this effect did not reach the significant level until five days later. Urine excretion was dramatically reduced after the rabbits were exposed to the diet for eight to nine days. The serum Na level was not significantly altered nine days after the diet. In contrast, the serum K level significantly decreased after the same length of time adapted to the diet.

7.3.2 Protocol 2

Because the majority of our experiments were performed in the rabbits adapted on a low K diet (0.25% K, TD 87433) with normal amount of Na, to examine whether this diet causes hypokalemia, the rabbits were exposed on the diet averagely for 10 days. Under these conditions, serum Na concentration did not significantly changed (142.4 ± 1.8 mM). However, serum K level was significantly reduced (2.8 ± 0.1 mM).

7.4 Discussion

The primary purpose of the studies is to determine whether low salt and low K intake affect serum Na and K level and urinary output of these cations in our system. The dramatical reduction in Na intake for nine days did not significantly altered serum Na level. However, both urinary Na concentration and total Na excretion significantly decreased. In contrast to rabbit handling Na, the moderate restriction of K intake significantly reduced the serum and urinary K level and total K output. The serum K level was virtually same on the low K diet as compared with on low Na and low K diet, suggesting the hypokalemic effect by restriction of K intake is independent of Na intake. In addition, urine output increased upon adaptation to the low Na and low K diet because low Na intake contracts extracellular volume, and low K intake impair urine concentration mechanism. All of these data are consistant with the previous reports (Dahl et al, 1972; Garg et al, 1982; Kirkendall et al, 1976). No further attempts are made to explore the mechanism for explaination of these observations, because it is impossible to clearly explain this very complicate issue by the present amount of data.

Table 7-1. Effect of low Na and low K diet on excretion of urine, urinary Na, and K

Days on the diet	$U_{[Na]}$ (mM)	$U_{[K]}$ (mM)	U_V (ml)	T_{Na} (mEq)	T_K (mEq)
0	65.6±12.5	120.2±21.9	92.3±15.3	5.2±0.4	11.7±3.1
1	28.4±12.3	78.1±16.6	75.2±21.2	1.5±0.4	5.1±1.3
2	22.3±5.6	46.0±10.2	105.0±35.6	1.7±0.4	3.7±0.8
3	23.0±7.1	46.2±13.0	76.5±32.8	0.8±0.3	1.8±0.8
4	25.8±6.4	28.2±2.9	98.2±26.9	1.8±0.3	2.6±0.5
5	13.6±2.7	33.6±7.1	275.8±89.7	2.8±0.7	6.2±1.2
6	8.3±2.1	26.9±6.5	103.4±64.5	0.6±0.3	1.6±0.7
7	9.5±3.3	33.2±9.4	131.2±47.0	0.6±0.2	2.2±0.7
8	11.3±4.2	32.6±9.2	67.6±32.0	0.4±0.1	1.0±0.3
9	15.9±6.1	36.2±10.0	30.8±6.7	0.3±0.1	0.8±0.2

there was a tendancy in decrease in urinary Na level one day after the animals were exposed to the diet. Urinary Na concentration was significantly reduced from 2nd day on the diet (at least $p<0.05$), and no further reduction in Na level was observed for the entire period on the diet. However, the rabbits excreted K differently from Na. A significant reduction in urinary K level (at least $p<0.01$) was observed one day after the animals were on the diet, and a further reduction in K concentration (at least $p<0.05$) was observed four days after the rabbits were on the diet. The total urinary excretion of Na and K was significantly reduced one day after the diet (at least $p<0.01$). The total excreted Na and K were further reduced eight days after the diet because the urine output decreased. Moreover, there was a trend in increase in urine output after animals were adapted to the diet. However, this effect did not reach the significant level until five days later. Urine excretion was dramatically reduced after the rabbits were exposed to the diet for eight to nine days. ($n=5$).
$U_{[Na]}$ and $U_{[K]}$, the concentrations of urinary Na and K, respectively. U_V, urinary output. T_{Na} and T_K, total urinary excretion of Na and K, respectively.

Table 7-2. Effect of low Na and low K diet, and low K diet on serum Na and K level (in mM)

	Low Na and Low K diet (n=5)	
	Na level	K level
Basal	140.5 ± 0.7	4.8 ± 0.1
On the diet	140.4 ± 3.2	2.8 ± 0.2*
	Low K diet (n=5)	
On the diet	142.4 ± 1.8	2.8 ± 0.1

* $p<0.05$, vs the basal.

CHAPTER 8
SUMMARY AND CONCLUSIONS

Potassium is pivotal for a variety of physiological functions such as acid-base balance, metabolism, cell volume regulation, blood pressure control, and the electrical properties of both excitable and nonexcitable cells. The carrying out of these vital functions by potassium is critically dependent on K homeostasis which is primarily accomplished by kidneys in the long-term. Potassium excretion is independent of K filtration. The collecting duct is the final tuning segment determining the amount of K appeared in the urine. The cortical collecting duct is capable of secreting K and absorbing K. The balance between these two processes contribute largely to determination of net potassium excretion. During past several decades, the potassium secretory mechanism has been extensively studied and relatively well understood. However, potassium absorptive mechanism remains to be elucidated. The discovery of H-K-ATPase in the collecting duct is a novel approach to understand K absorption. The present dissertation attempts to characterize this pump, mainly assessed as SCH28080-sensitive ^{86}Rb lumen-to-bath efflux coefficient, in the *in vitro* perfused CCD of the rabbits with emphasizing the effects of acute peritubular acidosis (10% CO_2), luminal amiloride addition and Na removal, and peritubular ouabain on this enzyme. Additional investigations have been made to study hormonal regulation of this pump.

Because renal H-K-ATPase has been demonstrated to be involved in proton secretion, and metabolic acidosis stimulates ATP hydrolysis by this enzyme, our first serial of studies were designed to examine whether acute respiratory acidosis induced by the presence of peritubular 10% CO_2 and presumably intracellular acidosis stimulates Rb efflux, and whether this stimulation is dependent on the presence of functional H-K-ATPase. These studies also extended to investigate the roles of carbonic anhydrase, microtubules, intracellular Ca activity, calmodulin, and basolateral K conductance in these maneuvers. Time-control experiments demonstrated that perfusion time did not significantly affect transepithelial voltage and Rb efflux. However, when the CCD was exposed to 10% CO_2 for 60 minutes Rb efflux increased from 93.1 ± 23.8 nm sec^{-1} (5% CO_2 period) to 249 ± 60.2 nm·sec^{-1} (10% CO_2 period, $p<0.05$). When the experiments were repeated under identical conditions except for the presence of the H-K-ATPase inhibitor SCH28080, 10% CO_2 failed to increase Rb efflux (76.4 ± 15.1 nm·sec^{-1}, 5% CO_2 period vs 76.8 ± 13.3 nm·sec^{-1}, 10% CO_2 period). This suggests that the effect of 10% CO_2 is dependent on H-K-ATPase. To examine the time course of this effect we conducted additional experiments. We were not only able to reproduce the stimulatory effect of 10% CO_2 on Rb efflux, but also observed this effect at the earliest collection time (30-60 minutes after exposure to 10% CO_2). Again, SCH28080 (10μM) totally abolished the Rb response, suggesting that an existing H-K-ATPase mediates the stimulatory effect of 10% CO_2 on Rb efflux. However, whether *de novo* H-K-ATPase synthesis is involved in this process can not be ruled out by the present experiments. Carbonic anhydrase catalyzes hydration of CO_2 which result in decrease in intracellular pH. The decrease in

intracellular pH stimulates H-K-ATPase which is evidently regulated by acid-base balance. Therefore, it is logical to evaluate the role of carbonic anhydrase in response to 10% CO_2. After stimulation of Rb efflux by 10% CO_2, subsequent addition of 0.1 mM methazolamide failed to affect Rb efflux. However, simultaneous exposure to 10% CO_2 and methazolamide prevented the stimulation of Rb efflux (98.6 ± 14.1 $nm \cdot sec^{-1}$ vs 86.2 ± 16.5 $nm \cdot sec^{-1}$). In addition, methazolamide had no significant effect on Rb efflux when the CCD was perfused continuously in the presence of 5% CO_2. The data are consistent with the conclusions that carbonic anhydrase is not necessary for maintaining H-K-ATPase activity, but is required for activation of H-K-ATPase by 10% CO_2. What is the mechanism explaining these observations? Exocytotic insertion of the acidic vesicles containing proton pumps has been demonstrated to mediate the stimulation of proton secretion by 10% CO_2 in turtle urinary bladder which has many transport characteristics similar to the collecting duct. This exocytotic process is pH- and Ca-dependent, and decrease in pH resulting in increase in intracellular Ca activity stimulates this process. Acetazolamide increases intracellular pH and decreases intracellular Ca activity, which conceivablly inhibit insertion process. This exocytotic process is also dependent on the presence of functional calmodulin and microtubule. Current knowledge of the cellular mechanisms explaining the insertion of proton pumps in the turtle urinary bladder is that exposure to CO_2 decreases intracellular pH, this process is mediated by carbonic anhydrase, the decrease in intracellular pH increases intracellular Ca activity, Ca combinds with calmodulin in order to amplify its signal, the complex of Ca-calmodulin causes the rearrengement of microtubules, the rearrengement of microtubules translocates the proton pumps from cytosol

to the apical membrane. In view of these obsercations, we postulate that the stimulation of Rb efflux by 10% CO_2 depends on fusion of H-K-ATPase to the apical membrane of the cortical collecting duct. This hypothesis is consistant with the effect of methazolamide. After Rb efflux was stimulated by 10% CO_2 (presumably activated insertion process), subsequent addition of methazolamide failed to inhibit Rb efflux, whereas simultaneous exposure to 10% CO_2 and methazolamide abolished the effect of 10% CO_2 (presumably prevented insertion initiation by alkalinization of intracellular pH). However, the proton sources originated from methazolamide-insensitive pathway remain to be identified. Exocytosis of proton pumps in turtle urinary bladder is microtubule-dependent. To further test the insertion hypothesis, and also to examine whether insertion of H-K-ATPase in the cortical collecting duct shares the similar characteritics as the insertion of proton pumps in the turtle urinary bladder, we selectively manipulated several experimental conditions which were expected to influence the functions of intracellular calcium, calmodulin, and microtubules. As predicted, buffering intracellular Ca activity with MAPTAM and antaganizing calmodulin with W-7 prevented the stimulatory effect of 10% CO_2. Similarly to methazolamide, only simultaneous exposure to 10% CO_2 and colchicine blocked the stimulation of Rb efflux by 10% CO_2. Taken together, these studies suggest that the stimulation of Rb efflux upon exposure to 10% CO_2 is dependent on the fusion of H-K-ATPase to the apical membrane of the tubules, and that this exocytotic process is mediated by intracellular pH and Ca, calmodulin, and microtubules. The next question remaining to be answered is the basolateral K exit mechanism. A Ba-sensitive K conductance has been identified in the basolateral membrane of both principal and intercalated cell of rats. If a similar

pathway is also present in the basolateral membrane of K-restricted CCD of rabbit, this pathway may mediate the effect of 10% CO_2. To test this hypothesis, the tubules was perfused in the presence of peritubular barium. Pretreatment with Ba totally blocked the stimulation of Rb efflux by 10% CO_2 (73.0 ± 8.2 nm·sec^{-1} vs 70.9 ± 8.3 nm·sec^{-1}), implying the efect of 10% CO_2 is dependent on the presence of functional barium-sensitive basolateral exit mechanism.

Amiloride substantially increases K permeation by normal and DOCA-treated CCD. This effect has been eluciated to be dependent on Ba-sensitive K conductance. To examine whether the effect of amiloride is persistant in the K-restricted CCD, the first set of experiments in this set of studies was performed in the absence of luminal Ba. Luminal addition of amiloride (1 mM) significantly increased the ^{86}Rb lumen-to-bath efflux coefficient from 90.7 ± 14.3 nm·sec^{-1} to 119 ± 20.6 nm·sec^{-1} ($P < 0.05$). Because 2 mM Ba has been shown to sufficient enough to block Ba-sensitive pathway, the second set of experiments was repeated under identical conditions except for the presence of 2 mM Ba. The effect of amiloride was fully blocked by the presence of Ba, suggesting that, like in normal and DOCA-treated CCD, a Ba-sensitive K conductance mediates the stimulation of K_{Rb} by amiloride. Ouabain has been demonstrated to increase K absorption in gastric gland. This effect has been attributed to gastric H-K-ATPase. The second purpose of this serial of studies is to examine whether the effect of ouabain is reproducible in the cortical collecting duct. Addition of 0.1 mM ouabain to the bath increased K_{Rb} (69.8 ± 11.1 nm·sec^{-1} vs 95.9 ± 18.7 nm·sec^{-1}, $P < 0.05$). However, this effect of ouabain on K_{Rb} was totally abolished by perfusion of the CCD in the presence

of luminal SCH28080 (10 μM) (69.8 ± 14.0 nm·sec^{-1}, basal period; 69.6 ± 14.1 nm·sec^{-1}, ouabain period), indicating that, similar to gastric gland, basolateral ouabain addition stimulates Rb (K) absorption via H-K-ATPase mechanism. The issue whether luminal Na-K-ATPase or luminal H-K-ATPase mediates K absorption has not been completely solved yet. Therefore, the third set of experiments of these studies aims at determination of the role of a primary active K pump in K absorption. We examined the action of known inhibitors of H-K-ATPase (SCH28080) and Na-K-ATPase (ouabain) on KRb. Luminal SCH28080 (10 μM) significantly reduced K_{Rb} by 39 ± 8.0% ($P < 0.05$), whereas luminal ouabain (0.1 mM) reduced K_{Rb} by 10 ± 14% (P = NS), suggesting that a luminal H-K-ATPase, not a luminal Na-K-ATPase, mediates Rb efflux.

Removal of luminal Na produces the similar effect on ion transport as amiloride addition in many cases. It is reasonable to examine the effect of lumen Na removal on Rb efflux. As predicted, Rb efflux was significantly enhanced by this maneuver. However, this effect was not abolished by luminal Ba. Only simultaneous addition of luminal Ba and SCH28080 (10 μM) fully inhibited the increase in KRb upon luminal Na removal (68.8 ± 9.8 nm·sec^{-1} vs 73.3 ± 10.0 nm·sec^{-1}, P = NS), indicating that the effect of luminal Na removal is more generalized than amiloride, and mediated in part via H-K-ATPase. To exploit these observations further, additional experiments were conducted to examine the effect of SCH28080 on Rb efflux in the presence and the absence of luminal Na. In the presence of luminal Na, SCH28080 (10 μM) reduced K_{Rb} by 15 ± 5.0%. However, in the absence of luminal Na, SCH28080 (10 μM) decreased Rb efflux by 48 ± 8.2%. The percentage inhibition of Rb efflux by SCH28080 was significantly greater in the absence of luminal Na than

in the presence of luminal Na ($P < 0.01$), suggesting that the presence of luminal Na reduces Rb (K) absorption by an H-K-ATPase-mediated mechanism. These observations are consistant with the hypothesis that Na may compete with K for transport via an H-K-ATPase. To test whether Na acts as a partial agonist of the H-K-ATPase we examined whether Na could be transported by H-K-ATPase. In the presence of luminal amiloride and an ambient K concentration of 0.5 mM, SCH28080 (10 μM) significantly inhibited the ^{22}Na lumen-to-bath efflux coefficient (K_{Na}) from 47.6 ± 4.8 nm·sec^{-1} to 35.0 ± 6.8 nm·sec^{-1} ($P < 0.005$), whereas the effect of SCH28080 on Na efflux was abolished when similar experiments were performed in the presence of 20 mM ambient K (33.3 ± 5.0 nm·sec^{-1} vs 30.6 ± 5.2 nm·sec^{-1}, P = NS). The lack of effect of SCH28080 on Na efflux in the presence of 20 mM K is not attributable to the lack of inhibitory effect of SCH28080 on H-K-ATPase, because SCH28080 significantly reduced Rb efflux when the experiments were repeated under exactly same conditions. Moreover, in the presence of luminal 1 mM amiloride, 10 μM SCH28080 significantly decreased KRb from 91.2 ± 10.2 nm·sec^{-1} to 68.3 ± 8.9 nm·sec^{-1} ($P < 0.05$). These data suggest that Na competes with K for transport via the H-K-ATPase, and H-K-ATPase is pharmacologically distinguishable from a Na-H antiporter.

In the gastric pland, the stimulatory effects of acetylcholine and gastrin on the gastric H-K-ATPase are mediated by intracellular Ca. Angiotensin II increases intracellular Ca. Angiotensin II binding has been demonstrated in the collecting duct. Moreover, angiotensin II has been shown to be antikaliuretic in human when infused at the concentrations that do not elicit systemic or renal hemodynamic changes. In view of these observation, we hypothesized that

angiotension II was the signal activating the renal H-K-ATPase. However, the experimental results did not prove the hypothesis with the serendipitous discovery that Ang II reduced Rb efflux, and this effect was dependent on a Ba-sensitive K conductance. The renal H-K-ATPase has been demonstrated to be similar to gastric H-K-ATPase pharmacologically and molecular biologically. Because histamine and acetylcholine are the potent stimulator of gastric H-K-ATPase, it is reasonable to investigate whether these observations are reproducible in the cortical collecting duct. Under our experimental conditions, neither histamine nor carbachol stimulated Rb efflux or net K absorption, suggesting that histamine and acetylcholine are not the first messager for renal H-K-ATPase.

In conclusion:

1. Exposure to 10% CO_2 dramatically stimulated Rb efflux within 30 minutes, and this effect was totally blocked by luminal SCH28080, suggesting that an existing H-K-ATPase mediates the stimulatory effect of peritubular acidosis on Rb efflux;
2. After Rb efflux was stimulated by 10% CO_2, subsequent addition of methazolamide did not inhibit Rb efflux, whereas simultaneous exposure to 10% CO_2 and methazolamide abolished the effect of 10% CO_2, implying that carbonic anhydrase is not necessary for maintaining H-K-ATPase activity, but is required for activation of H-K-ATPase by 10% CO_2;
3. Only simultaneous exposure to 10% CO_2 and colchicine prevented the stimulation of Rb efflux by 10% CO_2, reflecting that activation of H-K-ATPase depends on intact microtubule function;

4. Pretreatment with MAPTAM and W-7 blocked the effect of 10% CO_2 on Rb efflux, suggesting that the changes in intracellular Ca activity and calmodulin mediate the effect of respiratory acidosis;
5. Pretreatment with peritubular Ba abolished the effect of 10% CO_2, indicating that the stimulation of Rb efflux is dependent on the presence of functional barium-sensitive basolateral exit mechanism;
6. Luminal barium inhibitied Rb efflux, indicating that Ba-sensitive K conductance is present in the K-restricted CCD;
7. Luminal Ba fully inhibited the stimulatory effect of amiloride on Rb efflux, suggesting that a Ba-sensitive K conductance mediates the effect of amiloride;
8. Basolateral ouabain stimulated Rb efflux, and this effect was totally inhibited by SCH28080, implying that the stimulation of Rb efflux by ouabain is via H-K-ATPase mechanism;
9. Only luminal SCH28080, not luminal ouabain, inhibited Rb efflux, suggesting that a luminal H-K-ATPase, not a luminal Na-K-ATPase, mediates Rb efflux;
10. Removal of luminal Na stimulated Rb efflux, and this effect was not totally inhibited by SCH28080 or Ba alone, only simultaneous presence of these two agents fully blocked the effect oonducf Na removal, indicating that both Ba-sensitive K conductance and H-K-ATPase mediate the enhancement of K_{Rb} following lumen Na removal;
11. SCH28080 reduced Na efflux in the presence of low ambient K concentration, whereas the effect of SCH28080 was inhibited by high

ambient K level, suggesting that Na competes with K for transport via the H-K-ATPase, SCH28080 significantly reduced Rb efflux in the presence of 20 mM K, indicating that 20 mM K does not demolish the effect of this agent on renal H-K-ATPase;

12. Effect of SCH28080 on Rb efflux was demonstrable in the presence of maximal pharmacological dose of amiloride, implying that H-K-ATPase is pharmacologically distinguishable from a Na-H antiporter;
13. Angiotensin II inhibited Rb efflux, and this effect was prevented by Ba, indicating that Ang II inhibits Ba-sensitive K conductane;
14. Neither histamine nor carbachol stimulated Rb efflux or net K absorption, suggesting that histamine and acetylcholine are not the first messagers activating renal H-K-ATPase.

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BIOGRAPHICAL SKETCH

Xiaoming Zhou was born in Shanghai city, China. He obtained his Bachelor of Medicine degree with honor from Nanjing College of Traditional Chinese Medicine in February of 1982 and subsequently practiced pharmacy for two-and-half years. In August of 1984 he entered the graduate program in biochemistry at China Pharmaceutical University and was directed by Dr. Q. Chen, studying the inhibitory effect of anthraquinone derivatives on Na-K-ATPase derived from the rabbit renal medulla. In July, 1987, he was awarded a Master of Science degree in biochemistry. He entered the United States as a graduate student in the Department of Physiology, College of Medicine at University of Florida in October, 1987. Since matriculation of his primary interest has been the mechanisms of renal potassium transport, especially the renal H-K-ATPase, a K absorptive pump recently described by Dr. Charles S. Wingo, he formally joined Dr. Wingo's laboratory in January of 1988 to study this enzyme using primarily the technique of *in vitro* microperfusion. His research work has been recognized with several awards both regionally and nationally. He anticipates receiving his Ph.D. degree in physiology in May of 1992, and will persue the post-doctoral training in the laboratory of Dr. Douglas M. Fambrough at The Johns Hopkins University studying cation-transporting ATPase at molecular level.

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